Coinfection dynamics of experimentally challenged channel catfish (*Ictalurus punctatus)*

by

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Abstract

Catfish farming is the largest sector of the U.S. aquaculture industry and is of paramount economic importance for Southern U.S. agriculture. Maintaining and improving catfish health is a primary concern for producers, and bacterial pathogens can cause large-scale losses in production ponds. *Edwardsiella ictaluri*, *Aeromonas hydrophila*, and *Flavobacterium columnare* are the most predominant bacterial pathogens causing mortality within catfish production facilities. Interestingly, disease outbreaks resulting in high mortalities may also be coupled with multiple pathogens. Bacterial coinfections may often go unreported or misdiagnosed, resulting in a lack of proper mitigation for the coinfective effectors. Bacterial coinfections may increase the severity of the constituent pathogens along with grossly increasing mortality, thus creating economic losses. To assess and characterize the effects of bacterial coinfections, two pathogen challenge trials were conducted to compare in vivo virulence and fish immune responses resulting from exposure to single and coinfective bacteria. Trial results emphasize the importance of evaluating co-infections and demonstrate dramatic increases in mortality when two pathogens are combined, even at half-doses. The synthesis of these mortality and health metrics will aid fish health diagnosticians and channel catfish producers in developing therapeutants and prevention methods to control bacterial co-infections better.

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BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
cDNA	Complementary deoxyribose nucleic acid
CPM	Cumulative percent mortality
DNA	Deoxyribonucleic acid
DPC	Days post challenge
HPC	Hours post challenge
ESC	Enteric septicemia in catfish
MAS	Motile *Aeromonas* septicemia
MSA	Modified Sheih agar
MSB	Modified Sheih broth
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions per minute
TSA	Tryptic soy agar
TSB	Tryptic soy broth
US	United States
vAh	Virulent *Aeromonas hydrophila*

Chapter 1:

Literature Review

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1.1 Abstract

Catfish production is a major aquaculture industry in the United States and is the largest sector of foodfish production. As producers aim to optimize production yields, diseases caused by bacterial pathogens are responsible for high pond mortality rates and economic losses. The major bacterial pathogens responsible are *Edwardsiella ictaluri*, *Aeromonas* spp., and *Flavobacterium columnare*. Given the outdoor pond culture environments and ubiquitous nature of these aquatic pathogens, there have been many recent reports of co-infective bacterial infections within this aquaculture sector. Co-infections may be responsible for altering disease infection mechanics, increasing mortality rates, and creating difficulties for disease management plans. Further, proper diagnoses of primary and secondary pathogens are essential in ensuring the correct treatment approaches for both antimicrobials and chemical applications. A thorough understanding of the interactions and infectivity dynamics for these warmwater bacterial pathogens will allow for the adoption of new prevention and control methods. This review aims to provide an overview of co-infective pathogens in catfish culture, along with present diagnostic case data from both Mississippi and Alabama to better define prevalence for these multiple-species infections.

1.2 Catfish culture in the southern United States

Aquaculture provides safe and sustainable fish crop for consumers, supplying approximately 490,041 tonnes of total aquaculture production per year in the United States. Of this total, 65 percent of production originates from finfish alone. Most common production species include catfish, trout, salmon, and tilapia [1]. Catfish farming is the largest aquaculture industry within the United States, producing half a billion dollars per year. Nearly, 300 million pounds of channel and hybrid catfish are produced annually, making it substantially larger than any other aquaculture industry. Production of freshwater catfish is dominated by the southeastern region of the United States. Top regions of production include Mississippi, Alabama, and Arkansas [2]. The Mississippi Delta's economy relies heavily on the revenue generated through the catfish industry and Mississippi's catfish industry produced $223,972,000 in annual sales with Alabama producing $98,763,000.

Catfish typically inhabit freshwater streams but are also found in brackish muddy waters, lakes, and ponds, which allows for them to be relatively tolerant to culture in the Southern united states. The optimal growth temperature for channel catfish is around 29.4°C and environmental temperatures can dictate appetite (increased temperatures lead to increased feed consumption to a limit, which in turn may influence growth mechanics). Freshwater catfish are primarily produced in ponds dug into the earth and the pond types often include levees and/or watershed designs [3].

Catfish culture systems differ between the two major catfish producing states in the U.S., with Alabama having mainly watershed ponds, while Mississippi has levee ponds or split-pond production systems with groundwater wells used to fill ponds. Initially, eggs are transferred to hatchery troughs following spawning. When eggs hatch, the fry are transported and placed into production ponds where they grow to fingerling sizes. Fingerlings are then subjected to grow-out and are harvested once a marketable weight (approximately 1½ lbs) is reached [4].

Though channel catfish are the most common catfish produced in the USA, production of hybrid catfish has been increased by producers due to higher survival rates, crop yields, resistance to certain pathogens, and health benefits [5,6]. Hybrids are produced by artificially breeding female channel catfish (*Ictalurus punctatus*) with male blue catfish (*Ictalurus furcatus*). Female channel catfish are injected with hormones to prompt ovulation and sperm is removed from male blue catfish and used to fertilize eggs. Inconsistent egg quality and poor hatchery conditions increase the difficulty of hybrid production and hybrid fry production costs [5]. However, genetic advances have been made resulting in the increased efficiency of hybrid fry production. Though hybrid catfish have presented traits of increased resistance to pathogens compared to channel catfish disease is still a concern for both [7]. Methods for controlling diseases have included, medicated feed, improved water quality, vaccines and genetic improvement [7]. For both channel and hybrid catfish, disease is a primary concern for farmers leading to further investigation into disease pathogens

and co-infections in order to mitigate the proper disease management and treatment practices. As such, an understanding of the primary disease mechanisms for common bacterial pathogens in catfish culture is essential to being able to discern dynamics related to bacterial co-infections.

1.3 Bacterial pathogens commonly observed in catfish culture

Due to the economic impact of the catfish industry in the southeastern United States, maintaining catfish health is the primary concern for most farmers. Overcrowding and elevated temperatures facilitate disease within the hatcheries and ponds, and results in high mortality rates along with profit declines due to mortalities as well as costs associated with treatment. The majority of disease-related deaths in the catfish industry originate from bacterial diseases. The most prominent being *Edwardsiella ictaluri, Flavobacterium columnare, and Aeromonas hydrophila* in channel catfish; 78.1% of production operations and 42.1% of ponds experience outbreaks of ESC and columnaris [8]. Each pathogen is responsible for causing substantial catfish mortalities and creating interruptions to production due to lost feeding time (growth) and mitigation with chemical or antibiotic treatment means (economic expenses). Extensive efforts from researchers have been devoted to the development of vaccines and other methods to reduce the losses caused by bacterial infections.

Losses mentioned due to each pathogen have been during single infections. Though often unreported, co-infections amongst these pathogens have increased mortality and created drastic economic losses. Within this sector, the mechanics and prevalence of these bacterial co-infections remain poorly documented. In order to properly diagnose and investigate co-infective factors involving these primary bacterial pathogens, it is important to comprehend disease etiology and current treatment options available. Improvements in treatment methods against single pathogen infections have increased over the years, yet many treatment effects have not been tested during mixed infections.

Edwardsiella ictaluri

Edwardsiella ictaluri is the causative pathogen of enteric septicemia (ESC) which is an extremely fatal disease. Approximately $60 million dollars have been attributed to losses due to ESC [9] and up to 47 percent of cases each year are from ESC [10]. *Edwardsiella ictaluri* enters through the gut and passes from the stomach into the intestine. While passing through the epithelial barriers, propria macrophages engulf *E. ictaluri.* Typically, macrophages aid the host in resisting the pathogen, yet *E. ictaluri* has been observed within macrophage vacuoles, leading to the finding that it can survive within the macrophage and spread through the bloodstream [9]. Once in the bloodstream, the pathogen moves to the kidneys [10]. Clinical signs of ESC include petechial hemorrhaging around pectoral fins and belly, white pustules in the liver,

exophthalmia, distended abdomen related to ascites fluid, and cranial ulcerations. Fish can be seen swimming in spiral motions along with swimming at the surface due to a systemic infection and inflammation of the brain. Pathogen diagnosis includes isolating *E. ictaluri* from internal organs, kidney, or spleen, on tryptic soy agar with sheep blood. [10]. Environmental elements are key in facilitating infection. Outbreaks are typically associated with prominent levels of stress, due to handling and confinement, and temperature. For infection to occur, the temperature must be within 22 °C- 28 °C range . Transmission takes place once an infected fish sheds the pathogen, thus allowing surrounding fish to ingest *E. ictaluri* [10]. This disease can manifest in catfish of varying size and age class, including fish that are of market size [9]. Although all ages of catfish can experience ESC, channel catfish fingerlings are most susceptible. Altogether, post-hatch losses due to disease typically arise from enteric septicemia [11]. ESC survivors possess immunity to the disease, rendering older catfish more resistant to reinfection. In addition to *E. ictaluri* infections in catfish, other related and pathogenic *Edwardsiella* spp. are also routinely isolated from catfish ponds, including *E. tarda* [12] and *E. piscicida* [13].

The management strategy and treatment for ESC often calls for feed medicated with antibiotics, including: sulfadimethoxine-ormetoprim, and florfenicol. There are several limitations when using medicated feed to treat. Medicated feed is expensive and sick fish often refuse feed, thus rendering treatment ineffective [11]. Other methods include restricting feed to the catfish while temperatures are within ESC's

optimal growth range. However, this method also has some limitations. Though it slows the spread, it can reduce growth of catfish during production [11]. Early diagnosis of ESC is vital in order to treat infections appropriately and effectively. Recently, several investigations have aimed to discern mechanisms of virulence for *E. ictaluri*. Abdelhamed et al. (2017) identified the TonB transducing system as a virulence factor that is intertwined with ESC pathogenesis [14]. The role of lipopolysaccharides (LPS) in *E. ictaluri* virulence has also been investigated, with selected mutations related to LPS biosynthesis resulting in such modifications as altered biofilm formation abilities and motility [15]. An understanding of these virulence factors is of great importance for understanding the associated pathogenesis of ESC, and recently [16] identified differences in plasmids and virulence factors in *E. ictaluri* isolates from various fish species. Thus, the infectivity of *E. ictaluri* still remains a topic of research interest to more thoroughly define mechanisms of entry and host-pathogen interactions.

The development and introduction of ESC vaccines has been proven to decrease infection rates, but it does not eliminate threats of infection. Typically, ESC vaccines have been administered through immersion baths on catfish fry [11]. However, his method may not be the most effective as fry may not be immunocompetent. It has been documented that immunoglobulin responses cannot appear in channel catfish until 3 to 4 weeks post hatch [17]. Therefore, fingerling vaccination is likely the most

effective method. For catfish aquaculture, there are limited opportunities to vaccinate fingerlings using immersion delivery, therefore, effective oral vaccines are preferred.

Live *E. ictaluri* vaccines have received considerable attention due to the lack of immune response elicited from bacterin vaccines. Live vaccines have the ability to generate a multitude of immune responses, thus allowing higher protective abilities than the killed vaccines [18]. A live-attenuated oral vaccine has been developed to combat the pathogen. The vaccine strain (S97-773-340X2) was attenuated by passage on medium containing increasing concentrations of rifampicin, a method previously reported by Klesius and Shoemaker (1999) [19]. The effectiveness of the live-attenuated ESC vaccine was tested through the use of laboratory and experimental pond trials [20] and the results demonstrated significant increases in fingerling survival [21]. Commercial feed was diluted with the 340X attenuated isolate and administered to fingerling catfish orally [22]. Increased feed consumption of vaccinated fingerlings was documented along with a decrease in mortality due to disease compared to nonvaccinated fish [20]. In addition to survival, antibody responses were also measured. Vaccinated fish presented with an 18-fold increase in anti-*E. ictaluri* antibody levels when compared to nonvaccinated fish [20]. Antibody production is correlated to protective abilities against certain pathogens [23]. Elevated anti- *E. ictaluri* antibody levels present in vaccinated fish show an increase in immune response, demonstrating the vaccine's ability to hinder disease spread [20]. The results from the live-attenuated ESC vaccine experiments and pond trials indicated a

live-attenuated oral vaccine is proven to be an effective avenue to protect and immunize fingerling channel catfish [20].

In addition to vaccination, the use of hybrids has reduced the impact of *E. ictalurid* infections on the industry. Hybrid catfish have demonstrated considerable resistance to ESC, thus promoting their use to farmers and researchers. Blue catfish have also been shown to have a higher tolerance to ESC. Analyzing the resistance of multiple catfish species and families [24] has become increasingly important providing the possibility for farmers to raise hybrids by taking advantage of hybrids ability to have increased resistance to disease [9].

Virulent Aeromonas hydrophila

Historically, *A. hydrophila* has been considered a secondary pathogen in fish production, with cases of motile Aeromonad Septicemia (MAS) typically observed in fish that are stressed due to adverse environmental conditions or infection by a primary pathogen [25]. Fish with MAS can exhibit an array of symptoms, especially hemorrhaging and lesions that can progress to necrotic ulcers, and MAS is associated with high fish mortality [26]. It is common to co-isolate *A. hydrophila* and other pathogens from fish suffering from MAS symptoms, including such pathogens as *E. ictaluri, F. columnare* or *Vibrio parahaemolyticus* [27;28]. There is significant antigenic diversity among *A. hydrophila* strains, with 44 different O-antigen serotypes observed among mesophilic *A. hydrophila* strains [29], but more recent description of

A. hydrophila strains isolated from diseased fish are not commonly serotyped. The diversity of bacteria within the *A. hydrophila* complex that infect fish [30] and the ubiquitous presence of *A. hydrophila* within aquatic ecosystems particularly in biofilms and sediments [31] pose significant challenges for the generation of fish vaccines that are broadly protective against *A. hydrophila*.

A significant challenge for fish farmers has been the emergence of a hypervirulent pathotype of *A. hydrophila* causing MAS in farmed carp species in China first reported in 1989 [32], and in farmed catfish in the southeastern United States first reported in 2009 [33]. Fish infected with hypervirulent *A. hydrophila* (vAh) experience rapid onset of MAS disease and very high mortality. This virulent strain has been estimated to have caused greater than 12 million dollars in economic losses since the fish appearance in the U.S. industry [9,34].

The development of vaccines to protect farmed fish against *A. hydrophila* has been reported beginning in the 1970s. The strategies to vaccinate fish include the use of bacterins (inactivated cells), live-attenuated bacteria, and recombinant vaccines. *A. hydrophila* bacterins prepared by formalin- or heat-inactivation were reported to protect channel catfish [35], walking catfish (*Clarias batrachus L.*) [36], goldfish (*Carassius auratus*) [37], rainbow trout (*Oncorhynchus mykiss*) [38], carp (*Cyprinidae*) and loaches (*Misgurnus*) [39]. These bacterins triggered strong adaptive immune responses and have been shown to stimulate *A. hydrophila*-specific antibody titers and peroxidase activities in walking catfish [36], goldfish [37], and rainbow

trout [38]. Likewise, *A. hydrophila* bacterins have been observed to increase the expression of immune-related functions like IgM, IL-10 and lysozyme in carp and loaches [39].

The use of live-attenuated *A. hydrophila* vaccines can trigger more intense and prolonged immune responses by introducing avirulent bacteria by intraperitoneal injection [40], as has been demonstrated in common carp (*Cyprinus carpio L*) [41] and in Indian major carp species (*Catla catla, Labeo rohita, Cirrhunas mrigala*) [42]. The specific antibody titer in common carp was significantly increased by vaccination with live-attenuated *A. hydrophila* compared to fish that were vaccinated with formalin-killed vaccine [41]. For Indian carp species, vaccination with a hemolysin-negative *A. hydrophila* mutant induced significant protection (RPS >80%) and good agglutinating antibody response against virulent *A. hydrophila* [42].

Recombinant vaccines have been evaluated for their ability to protect rohu (*Labeo rohita*) [43], and American eel (*Anguilla rostrata*) [44] against *A. hydrophila*. *Escherichia coli* has been used overexpress pathogen genes, such as the outer membrane protein gene of *A. hydrophila,* which resulted in stimulation of IgM levels, lysozyme and significantly reduced fish cumulative mortality rates when challenged by *A. hydrophila* [43][44].

Experimental challenges with vAh strains isolated from US catfish result in significant mortality rapidly and the vast majority of fish that succumb to disease die within 24 hours [45]. The structure of the group 4 capsular polysaccharide and LPS-

associated O-antigen from a vAh strain isolated from a US catfish has a novel structure [46] unlike that of other *A. hydrophila* isolates from fish and a capsular polysaccharide-exporting mutant of the well-characterized catfish vAh isolate ML09-119 was observed to be attenuated in its virulence [47]. A catfish-derived vAh strain selected for resistance to two antibiotics demonstrated significant protection in channel catfish and Nile tilapia when IP-injected, resulting in 86-100% protection relative to naïve fish [48]. Attenuated vaccines to protect fish from vAh have been generated by selecting for antibiotic resistance [49], or by generating attenuated vAh via multiple gene deletions [50]. An attenuated vAh strain resistant to both novobiocin and rifampicin was used to vaccinate channel catfish by IP, resulting in 100% protection against the parent vAh strain with evidence for a strong antibody-mediated response and induction of Na(+)/K(+) ATPase α subunit, hepcidin, interleukin-1β and lysozyme c within the anterior kidney in vaccinated fish relative to naïve fish [49]. In a study that deleted five vAh genes (*aerA, hly, ahp, alt* and *ast*) to produce an attenuated mutant strain, this was able to elicit a strong adaptive immune response in grass carp (*Ctenopharyngodon idella*), and yielded a RPS of 70 or 75% when fish were vaccinated by immersion and subsequently challenged by two different vAh strains, or a RPS of 75 or 85% when fish were vaccinated by intracelomic injection and challenged by vAh strains [50].

Besides using attenuated vAh strains, the extracellular proteins (ECPs) can serve as antigens that can elicit a protective response in channel catfish [51;52]. The

antiserum from the vaccinated fish agglutinated both vAh cells and more than 68 pathogenic proteins were recognized and aggregated by catfish IgM, including aerolysin and hemolysin. All channel catfish immunized with vAh ECP (2 micrograms) and Freund's adjuvant by intraperitoneal (IP) injection survived challenge whereas naïve fish injected with adjuvant alone all died within five hours [51]. Furthermore, ECP-immunized sera from channel catfish could be used to passively immunize channel catfish and provide an RPS of 85% by two days post-vaccination [52].

The infection of vAh significantly induced transcription of apolipoprotein A1 [53], chicken-type lysozyme [54], G-protein coupled receptor 18 [55], and goose-type lysozyme [56] in kidney, liver, and other tissues of channel catfish. These proteins were then expressed in the *E. coli* expression system and exhibited high lytic activity against the pathogen. The recombinant vaccines provided 100% protection to catfish two days after IP injection, and the protection remained at 77% - 100% two to four weeks post-vaccination when challenged by vAh AL09-71 [28,29,30,31] Similar approaches using recombinant expression of vAh-derived aerolysin, hemolysin [57], ATPase [58], fimbrial proteins [59], immunogenic outer membrane proteins [60], aerA (hemolytic and cytolytic factor) [61] were used to vaccinate channel catfish, resulting in 58% - 98% protection relative to naïve fish. Collectively, these studies demonstrate that multiple vaccination strategies can be effective in providing protection against vAh to farmed fish. Ultimately, the vaccination strategy adopted by

fish producers will need to provide long-lasting adaptive immunity and be cost-effective in order to be widely used. An important question to be addressed by future research is whether any of the vaccines developed to protect fish against vAh strains will provide immunity against other *A. hydrophila* types that are ubiquitous in aquatic ecosystems.

Flavobacterium columnare

Flavobacterium columnare is the causative agent of columnaris disease with a worldwide distribution. The disease was first described in the early 1900's in the state of Iowa (USA), in which a thorough investigation of the disease was conducted [62]. Examination of infected tissue under a microscope revealed the responsible bacterium tended to form columns or haystacks; thus, the names *Bacillus columnaris* and columnaris disease were proposed for the bacterium and disease, respectively [62]. *Bacillus columnaris* was first cultured in 1944 [63] on low nutrient media and was reclassified as *Chondrococcus columnaris*. The bacterium has been reclassified several times as *Cytophaga columnaris* [64], *Flexibacter columnaris* [65], and finally, as *F. columnare* [66]. Research has revealed a striking degree of genetic variation among isolates of *F. columnare*, and phylogenetic analyses has defined four distinct genetic groups within the species [67]. Not only do these groups differ at the genomic level, but also in host associations. Genetic group 1 isolates are predominantly

associated with disease in salmonids and genetic group 4 isolates are associated with disease in tilapia (*Oreochromis* spp.) aquaculture [67].

In the US catfish industry, columnaris disease is the second leading cause of mortality. In East Mississippi and West Alabama alone, yearly losses attributed to *F. columnare* range between 1.5 and 2.4 million pounds for each region [68] (Bill Hemstreet, Alabama Fish Farming Center, Greensboro, AL USA, personal communication). Mortalities in extreme cases have reached 90%, and in commercial ponds mortalities have reached 50-60% [9] resulting in $30 million dollars in economic losses. Columnaris disease has a bimodal distribution with most cases occurring in the spring and fall, at a time when pond temperatures are changing; however, recent increases in cases during the summer months has been noted. Clinical signs include gill necrosis, fin rot, and skin lesions often with a yellowish color due to the pigmentation of *F. columnare*. Infections may be exclusively external, internal (systemic) or a combination of both [27]. Juvenile catfish are more susceptible to columnaris disease; however, disease may occur during any phage of commercial production. Diagnosis of columnaris disease is achieved by observation of clinical signs, presence of long slender gram-negative rods in wet mounts of affected tissues, and isolation of *F. columnare* colonies from tissues characterized as adherent to agar, yellowish in color, and rhizoid colony morphology. Of importance is the common documentation of co-infections upon examination of columnaris disease cases. Hawke

and Thune (1992) examined 99 bacterial disease cases in catfish and greater than 50% represented co-infections [27].

Effective and practical vaccination is highly desired by catfish producers to reduce the impact from columnaris disease. Early research evaluated simple formalin-inactivated bacterins administered by immersion that showed some beneficial effects including reduced mortality and antibiotic use [69]. Subsequent research resulted in the commercialization of a live-attenuated vaccine that showed good efficacy in the laboratory [70]. Efficacy under production conditions was variable and use of the vaccine by the catfish industry declined [71]. As such, several new vaccine platforms have been assessed as mitigation tools, with many focusing on the outer membrane proteins (OMPs) as important, antigenic regions [72]. Further, a recombinant vaccine (comprised of *F. columnare* chaperone protein DnaK) was evaluated by [73] and showed promise for vaccine efficacy. Similarly, a new live-attenuated vaccine has been tested under conditions similar to production and the results demonstrated a beneficial effect including lower food conversion ratios and larger average weight at harvest [74]. However, there are currently no effective and commercial vaccines available for use in the catfish industry; thus, prevention of columnaris disease relies heavily upon using good pond management practices to reduce risk factors such as stress, handling, and poor water quality. Epizootics can and will occur with these in place and require treatment using approved antibiotics or other compounds.

1.4 Bacterial coinfections

Co-infections are frequently seen in nature and arise when two or more pathogens infect one host. Infections can occur from two primary pathogens infecting the host concurrently or one pathogen can develop as a secondary infection [75]. Mixed infections have also made determining the primary cause of mortality exceedingly difficult, thus increasing treatment difficulties. Though co-infections are so frequent in typical fish environments, associated information is scarce. The environment plays a principal role by facilitating microorganisms which can lead to co-infection. Co-infections can exist between bacterial pathogens, viruses, and parasites, allowing for a wide range of clinical manifestations and complications for treatment regiments in pond environments [75].

Parasites are frequently seen in combination with bacterial pathogens. Researchers have investigated whether parasites can act as vectors for bacterial pathogens increasing infection rates along with mortality. Parasites are known to increase host susceptibility by creating portals of entry for potential bacterial pathogens resulting in high mortality and stress. However, their ability to act as vectors is unknown. *Bolbophorus damnificus* is a parasitic trematode responsible for mortalities in commercial ponds in Mississippi. When co-infections between *B. damnificus* and *E. ictaluri* occur mortality rates have been documented to increase dramatically [76]. *B. damnificus* could potentially create a portal of entry allowing

higher host susceptibility to ESC. Similarly, proliferative gill disease (PGD) could present a portal of entry for bacterial infections through damage and hemorrhaging of the gill in channel catfish. PGD is a result from a myxozoan parasite, *Henneguya ictaluri*, causing branchial inflammation and the breakdown of chondrocytes [77]. Exposure of hemorrhaged gills due to PGD could allow *A. hydrophila* or other bacterial pathogens a route of transmission into the host's blood system. This *Aeromonas* spp. infection, in combination with PGD, substantially increases mortality rates thus increasing economic losses [77]. Simultaneous infections amongst these pathogens facilitates increased exposure to bacterial infections. Similarly, although not reported in catfish species, a combination of *A. hydrophila* and *Epistylus* spp. cause "red sore disease" has also become of recent interest for fish disease diagnostics in freshwater systems [78]. *Ichthyophthirius multifillis* (ich; white spot disease), a freshwater protozoan that causes high mortalities within the industry, has been investigated for its potential in acting as a vector for *E. ictaluri*. After investigation, evidence supported ich's ability to act as a vector, as researchers concluded transmission of bacterial diseases can be increased through parasitic vectors [79]. Yusoff et al. (2020) also reported *A. hydrophila* as a secondary pathogen to *Dactylogyrus* spp., attributing the external, parasite-yielded injuries to the bacterial sites of entry [80].

Information pertaining to dual bacterial infections is highly limited when compared to parasitic and bacterial co-infections. Bacterial co-infections are known to

cause drastic effects in increasing the extremity of other diseases along with grossly increasing mortality, changing host-susceptibility, and duration of infection [75]. Farmers frequently under report bacterial co-infections leaving little data related to outbreaks including diagnosis, immune response of host [75], and clinical signs. Clinical signs arising from co-infections can be difficult to distinguish due to lack of information on which pathogen is responsible for which sign of infection. Other infectious agents occurring concurrently with primary pathogens are often characterized as secondary infections or opportunistic, resulting in most of the research to be focused on primary pathogen infections. Co-infections change fish susceptibility to a variety of pathogens [75] resulting in outbreaks causing high mortality. Interactions between the pathogens can lead to bacterial load variability, where loads can be either both suppressed, increased, or one potentially suppressed while the other is increased, although, extraordinarily little information is known about how loads are affected during co-infections. Competition between resources of the host is typical in co-infections; modifying immune activity against other pathogens can suppress or increase the immune response leading co-infections to be either synergistic or antagonistic affecting and altering the host-pathogen interactions [75]. Antagonistic effects allow the primary pathogen to obstruct the secondary pathogen while synergistic effects create immunosuppressive effects allowing both pathogens to infect the host increasing mortality.

The co-infective ability of bacterial fish pathogens warrants further investigation to better comprehend natural exposure in production systems. In cobia (*Rachycentron canadum*), *Vibrio harveyi* and *Photobacterium damselae* have been used in experimental co-infective challenges and differences in mortality were observed in fish receiving multiple pathogens when compared to some of the single-pathogen treatment groups [81]. In rainbow trout, a co-infective pathogen challenge with novel Family *Flavobacteriaceae* isolates also showed increased mortality when compared to single-isolate treatments [82]. Similarly, systemic infection and ulcerative dermatitis was observed within farmed barramundi (*Lates calcarifer*) and the cause was attributed to co-infection with *Steptococcus iniae* and *Shewanella algae* [83]. A co-infection of *Yersinia ruckeri* and *Pseudomonas fluorescens* was also found to have caused mortality rates of up to 40% across three rainbow trout production farms in Turkey [84]. *Cyprinus carpio* var. *koi* (koi carp) experienced high mortality rates in Tianjin breeding farms. Moribund koi carps were cultured and *A. veronii* and *V. cholerae* were isolated and identified for the first time in combination by Han et al (2021) [85]. Both pathogens presented similar clinical signs including lesions along the liver, intestine, and spleen. Fish also exhibited intestinal hemorrhaging. Research indicated additional studies should be conducted to further study pathogenicity. This work could aid in developing treatment along with future prevention methods [85]. A study conducted by Chandrarathna et al. (2018) examined the effects of co-infection in zebrafish [86]. *Aeromonas hydrophila* and *A. veronii* were identified as the

causative pathogens inducing mortality amongst zebrafish. Isolates presented to be multidrug resistant. Once challenged, single infections with the pathogens caused less mortality than the co-infections suggesting that mixed infections of *A. hydrophila* and *A. veronii* have a higher pathogenicity than single infections [86].

To assess the full extent of co-infection outbreaks more specifically within catfish production, studies must be conducted to quantify multiple pathogens effects on host mortality. *Pangasianodon hypophthalmus* (striped catfish) were observed to have encountered natural infections of *E. ictaluri* in Thailand. Researchers discovered striped catfish were experiencing co-infections of *F. columnare* and *E. ictaluri*. The investigation into the outbreak aimed to characterize both single and dual infections from each pathogen. Clinical signs from both *E. ictaluri* and *F. columnare* were consistent between naturally occurring infections and induced infections [87]. Researchers were able to fulfill Koch's postulates and provided data of molecular markers to better identify outbreaks in fish. Similarly, striped catfish were immersed with both *E. ictalur*i and *A. hydrophila.* Results indicated the co-infection caused 95% cumulative mortality while the single infection of *E. ictaluri* only had 80% and *A. hydrophila* 10%, thus suggesting that *A. hydrophila* acted as a secondary or opportunistic pathogen [88]. Grizzle and Kiryu (1993) also found that channel catfish that were displaying latent *A. hydrophila* infection following experimental challenge also exhibited infections with *Acinetobacter* spp., *Plesiomonas* spp., and *Pseudomonas* spp.) [25]. Nofal and Abdel-Latif (2017) also reported a variety of

mixed bacterial, and mixed bacterial-parasitic infections in African catfish, with the prominent bacterial pathogens *Vibrio* spp., *A. hydrophila,* and *E. tarda* recorded from the pond fish kills [89].

In 2017, researchers at the E. W. Shell Fisheries Center at Auburn University (Auburn, AL) observed chronic mortalities in channel catfish within an in-pond raceways system. Mortalities were deemed unusual due to outbreaks occurring at lower water temperatures and during periods of reduced feeding. After further investigation, three different bacterial pathogens were isolated, indicating a co-infection. Gram-negative bacteria *A. veronii* and *S. putrefaciens* were identified along with the gram-positive bacterium *S. parauberis.* Fish were exposed to pathogens in an attempt to identify the primary causative agent. Both *A. veronii* and *S. parauberis* were unsuccessful in inducing mortality, while exposure to high doses of *S. putrefaciens* induced signs of disease and low mortality rates (33%-50%). Researchers concluded infection with *A. veronii*, *S. parauberis*, and *S. putrefaciens* was a novel co-infection, and future investigations should be done to determine transmission and pathogenicity of *S. parauberis* and *S. putrefaciens* [90].

Immune responses associated with co-infections must be further studied in attempt to develop future avenues of treatment and prevention [75]. The mucosal surface of catfish is an important immune component to investigate during co-infections. Mucosal surfaces of channel catfish are the first line of defense against pathogens thriving in aquatic environments [91]. Investigating fish mucus's innate

immune defense mechanisms can lead to better understanding of how pathogens attach and enter the host along with the aiding in developing prevention methods. *Flavobacterium columnare* is a prime example of a bacterial pathogen being dependent on attaching to the mucosal surface of the host in order to cause infection. Most studies regarding this are focused primarily with the liver, spleen, and kidney immune factors, however, by examining the expression patterns within the mucus researchers can determine whether attached bacteria suppress host immune responses [92]. The immune response of the mucosal surfaces of channel catfish have also been investigated during *A. hydrophila* infections. Vital lectins and proteins were observed to be altered potentially enhancing the pathogen's ability to disrupt and adhere to the mucosal barrier [92]. Though studies have been conducted to determine single pathogen effects on mucosal surface of catfish, similar studies have not been conducted to document multiple pathogen's effects.

1.5 Diagnostic summary of recent bacterial co-infections in Alabama and Mississippi

While diagnostic case records are fraught with submission bias, they can still provide valuable insight into the disease prevalence in the catfish industry. Bacterial diseases are the most commonly diagnosed diseases for catfish case submissions (each case submission is a composite sample of fish collected from a single pond on a given day) at the Alabama Fish Farming Center (AFFC) in Alabama and the Aquatic

Research & Diagnostic Laboratory (ARDL) in Mississippi. In addition, Mississippi and Alabama are the top producing catfish states in the US, each having farms that produce channel catfish and hybrid catfish (♀, *Ictalurus punctatus* × ♂, *I. furcatus*). The top prevalent co-infection patterns differ between these states, which may be a reflection of the different system types.

The AFFC records showed that cases of co-infection in Alabama were primarily the bacteria *F. columnare* and either *A. hydrophila* or various other *Aeromonas spp.*, which include *A. sobria, A. caviae,* and *A. veronii*. The next frequently recorded co-infections were between *E. ictaluri* and *F. columnare* (Table 2). However, the ARDL data revealed bacterial co-infections occurred more frequently among the two most commonly diagnosed bacterial diseases, ESC and columnaris disease (Tables 1 and 2). These co-infection trends are consistent year-to-year for the AFFC and ARDL from 2016 to 2020.

There are some consistent trends within the Alabama and Mississippi records. Channel catfish represent most of *E. ictaluri-F. columnare* co-infection cases and are at least twice, if not more, the number of hybrid catfish cases. Within these cases, it is difficult to assign with certainty which is the primary pathogen as each can cause disease by itself. While *F. columnare* is often thought to be secondary, columnaris disease is usually seen earlier in the year when the cooler temperatures are less conducive for *E. ictaluri* infections and may set the fish up for co-infections later. For *Edwardsiella piscicida*-columnaris disease co-infections, hybrid catfish represent the

majority of cases, but this is not unexpected since hybrid catfish are more susceptible to *E. piscicida* infections [93] but are more resistant to ESC [94] and columnaris disease [95]. However, this combination of bacterial diseases is much less common than *E. ictaluri-F. columnare* infections. Therefore, columnaris disease is likely a secondary infection based on the severity of *E. piscicida* lesions compared to the *F. columnare* lesions.

The ARDL data showed co-infections between *F. columnare-A. hydrophila* and *F. columnare-Aeromonas* spp. infections, the latter of which are cases where the species of *Aeromonas* could not be speciated by the BD BBLTM CrystalTM Enteric/Nonfermentor (Becton Dickinson and Company, Sparks, MD), were significantly lower than those reported in Alabama.

In 2017, there were five *Yersinia ruckeri* cases in hybrid catfish from one farm in Mississippi, one of which was a *Y. ruckeri*-columnaris disease co-infection. While typically considered a coldwater fish pathogen, *Y. ruckeri* can be occasionally seen in warmwater fish species, including catfish [96]. No *Y. ruckeri* co-infection cases were diagnosed in Alabama from 2016-2020.

1.6 Future directions for bacterial co-infection mitigation and research

A more comprehensive understanding of bacterial co-infections will present many new avenues for enhancements to fish health within catfish production. By further capturing mechanisms for infectivity and virulence and detailing predominant pathogens in diagnostic casework, treatment regimens may be more customized for enhanced efficacy. For instance, properly identifying primary and secondary pathogens will allow for the appropriate selection of antibiotic or chemical treatment means. As we have limited approved drugs for use in cultured fish species, judiciously administering antibiotics allows producers to retain treatment efficacy. Similarly, as we detail more information on the prevalence and dynamics of antibiotic sensitivity in aquaculture pathogens, the importance of profiling antibiotic susceptibility of multiple pathogen infections is clear. Additionally, if both the presence and role of co-infective pathogens is discerned, more rearing-related parameters (i.e., water quality, temperature, feed administration) may be manipulated to cater to the primary effector. Further discerning expanded treatment efficacy has major economic implications for catfish producers, as treating large ponds for diseases can be very expensive for chemical treatments (aside from medicated feed expenses).

With respect to research aims for co-infective bacterial pathogens, access to case diagnostic profiles (both on a small and meta-scale) will provide directions for strain selections that best characterize ongoing health concerns in production ponds. There is a need to establish more natural multi-pathogen *in vivo* challenge models that best

represent the role of both primary and secondary effectors. For instance, dose-concentration studies and timing of pathogen introductions during an *in vivo* challenge are important aspects of emulating natural conditions related to disease onset. From this data, further mechanism of infectivity and changes to pathogenesis during co-infection events can be discerned using molecular tools (i.e., gene expression and sequencing) and growth dynamics.

Further, the cross-protective ability of vaccines used in catfish culture is also of importance to multi-infection mitigation plans. Optimizing catfish vaccine to provide an expanded umbrella of protection will also potentially reduce bacterial co-infections through an enhanced immune system response and/or shared protective antibodies that are cross-reactive. Aside from chemical treatments and prophylactics, the ability to select genetic lines that are more disease resistant to selected bacterial pathogens would also be of benefit to catfish producers. Several catfish strains and types (i.e., genetic crosses or transgenics) have established evidence for some aspects of disease resistance, yet the expansion and determined scope of these resistance capabilities is of interest.

Co-infective bacterial pathogens in the catfish production sector are not well reported in the literature and warrant further investigation to fully characterize their pathogenesis in production systems. Through the advanced analysis of disease diagnostic data and expanded, targeted research aims, the role of co-infective bacterial pathogens may be further elucidated to better control pathogens in catfish aquaculture.

Tables

Table 1. Mississippi State University - College of Veterinary Medicine Aquatic Research & Diagnostic Laboratory - Stoneville, MS
Polymicrobial Cases from 2016-2020 Disease Diagnosis as a Percentage of Total Case Submissions

2020 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris; *Aeromonas hydrophila*							1						1	0.13	1			
Columnaris; *Aeromonas* sp.						2							2	0.26	*2*			
Edwardsiella piscicida: columnaris					6	5		2		1			14	1.83	2	12		
Enteric septicemia of catfish; *Aeromonas hydrophila*							1						1	0.13	1			
Enteric septicemia of catfish; columnaris			2	11	11	11	4	67	45	7	2		160	20.97	118	42		
Enteric septicemia of catfish; columnaris; *Aeromonas* sp.				1									1	0.13	1			
Total cases: 763																		
2019 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris, *Aeromonas hydrophila*,			1										1	0.14	1			
Columnaris, *Aeromonas* sp.					2	1							3	0.42		3		
Edwardsiella piscicida, columnaris,					2	2	1		2				7	0.97	1	5	1	
Enteric septicemia of catfish, columnaris					10	8	22	26	27	17			110	15.26	84	26		
Total cases: 721																		
2018 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris, *Aeromonas* sp.								1					1	0.2		1		
Edwardsiella piscicida(tarda), columnaris					2	1							3	0.5		3		
Enteric septicemia of catfish, *Aeromonas* sp.										1			1	0.2		1		
Enteric septicemia of catfish, columnaris				1	6	2	8	41	14	13	1		86	13.0	70	16		
Total cases: 660																		
2017 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris, *Aeromonas* sp			2		1								3	0.3	3			
Edwardsiella piscicida, columnaris,			1	1	1	2		1	4	1			11	1.3	1	10		
Enteric septicemia of catfish, columnaris				1	19	28	16	51	44	25	1	1	186	21.6	155	31		
Enteric septicemia of catfish, *Edwardsiella tarda*											1		1	0.1		1		
Yersinia ruckeri, columnaris			1										1	0.1		1		
Total cases: 861																		
2016 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	**%**	CH	HY	BL	OS
Columnaris, *Aeromonas* spp.				1			1	1					3	0.4	1	2		

Edwardsiella piscicida, columnaris				1		1	2	1	3	2	2		12	1.6	1	11		
Edwardsiella piscicida, columnaris, *Aeromonas hydrophila*								1					1	0.1		1		
Enteric septicemia of catfish, *Aeromonas hydrophila*										1			1	0.1	1			
Enteric septicemia of catfish, columnaris				2	13	10	11	21	28	23	1		109	14.7	79	30		
Total cases: 744																		

2

3

4

Table 2. Alabama Fish Farming Center Polymicrobial Cases from 2016-2020 Disease Diagnosis as a Percentage of Total Case Submissions

2020 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris; *Aeromonas hydrophila*		2	4	2	2	8		2	3	2			25	8.4	22	3		
Columnaris; *Aeromonas* sp.			2			5	3		2				12	4	11	1		
Edwardsiella piscicida: columnaris							1						1	0.3		1		
Enteric septicemia of catfish; *Aeromonas hydrophila*				1									1	0.3	1			
Enteric septicemia of catfish; columnaris			5		4	3	4	1	1	3			21	7	17	4		
Total cases: 306																		
2019 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris, *Aeromonas hydrophila*,				2	7	5	4	5	5				28	9.8	23	5		
Columnaris, *Aeromonas* sp.			5	10	1	1		4					21	7.3	14	7		
Edwardsiella piscicida, columnaris,							1						1	0.3		1		
Edwardsiella piscida, *Aeromonas* spp.							1						1	0.3	1			
Enteric septicemia of catfish, *Aeromonas hydrophila*								3					3	1	6			
Enteric septicemia of catfish, *Aeromonas* spp.								10					10	3.5	4	2		5
Enteric septicemia of catfish, columnaris							6	4	1				11	3.8	8	3		
Total cases: 287																		
2018 Polymicrobial Disease Diagnostic Cases																		
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris, *Aeromonas hydrophila*,			1		2					1	1		5	1.7	3	2		
Columnaris, *Aeromonas* sp.			4	10	1	5							20	6.8	10	10		
Edwardsiella piscicida, columnaris				3	2								5	1.7	1	4		
Enteric septicemia of catfish, *Aeromonas hydrophila*													0	0				

Enteric septicemia of catfish, *Aeromonas* sp.												0	0				
Enteric septicemia of catfish, columnaris				1	1							2	0.7	2			
Columnaris, *Pleisiomonas* sp.						1						1	0.3	1			
Total cases 296																	
2017 Polymicrobial Disease Diagnostic Cases																	
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	%	CH	HY	BL	OS
Columnaris, *Aeromonas hydrophila*,				1	1	1		1	4				8	2.3	5	3		
Columnaris, *Aeromonas* sp.		2	6	2	6								16	4.5	10	6		
Edwardsiella piscicida, Columnaris,										1			1	0.3		1		
Enteric septicemia of catfish, *A. hydrophila*													0	0				
Enteric septicemia of catfish, columnaris						1			3	1			5	1.4	5			
Enteric septicemia of catfish, *Edwardsiella tarda*, *Aeromonas* spp.													0	0				
Total cases 352																	
2016 Polymicrobial Disease Diagnostic Cases																	
Disease	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total	**%**	CH	HY	BL	OS
Columnaris, *Aeromonas hydrophila*,					1	5		3	3	1			13	2.8	6	7		
Columnaris, *Aeromonas* spp.			3	8	4		1			2			18	3.9	16	2		
Edwardsiella piscicida, columnaris													0	0				
Enteric septicemia of catfish, *Aeromonas hydrophila*						1				1			2	0.4	2			
Enteric septicemia of catfish, Ccolumnaris			1	1	1	1				11	1		16	3.5	13	3		
Enteric septicemia of catfish, *Aeromonas* spp.						2								0	2			
Streptococcus spp., *Aeromonas hydrophila*							1							0	1			
Columnaris, *Pleisiomonas* spp.					1									0	1			
Total cases: 460																	

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Chapter 2:

Infection Dynamics of Experimental *Edwardsiella ictaluri* and *Flavobacterium covae* Coinfection in Channel Catfish (*Ictalurus punctatus*)

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2.1 Abstract

Edwardsiella ictaluri and *Flavobacterium covae* are pervasive bacterial pathogens associated with significant losses in catfish aquaculture. Bacterial coinfections have the potential to increase outbreak severity and can worsen on-farm mortality. A preliminary assessment of *in vivo* bacterial coinfection with *E. ictaluri* (S97-773) and *F. covae* (ALG-00-530) was conducted using juvenile channel catfish (*Ictalurus punctatus*). Catfish were divided into five treatment groups: 1) mock control; 2) *E. ictaluri* full dose (immersion; 5.4×10^5 CFU mL^{-1}); 3) *F. covae* full dose (immersion; 3.6×10^6 CFU mL^{-1}); 4) *E. ictaluri* half dose (immersion; 2.7×10^5 CFU mL^{-1}) followed by half dose *F. covae* (immersion; 1.8×10^6 CFU mL^{-1}); and 5) *F. covae* half dose followed by half dose *E. ictaluri*. In coinfection challenges, the second inoculum was delivered 48 hours after the initial exposure. At 21 days post-challenge (DPC), the single dose *E. ictaluri* infection yielded cumulative percent mortality (CPM) of 90.0 ± 4.1 %, compared with 13.3 ± 5.9 % in the *F. covae* group. Mortality patterns in coinfection challenges mimicked the single dose *E. ictaluri* challenge, with CPM of 93.3 ± 5.4% for fish initially challenged with *E. ictaluri* followed by *F. covae,* and 93.3 ± 2.7 % for fish exposed to *F. covae* and subsequently challenged with *E. ictaluri*. Despite similarities in final CPM within the coinfection groups, the onset of peak mortality was delayed in fish exposed to *F. covae* first but was congruent with mortality trends in the *E. ictaluri* challenge. Catfish exposed to *E. ictaluri* in both single and coinfected treatments displayed increased serum lysozyme activity at 4-DPC ($P < 0.001$). Three pro-inflammatory cytokines (*il8, tnfa, il1β*) were evaluated for gene expression, revealing increased expression at 7-DPC in all *E. ictaluri* exposed treatments ($P<0.05$). These data enhance our understanding of the dynamics of *E. ictaluri* and *F. covae* coinfections in US farm-raised catfish.

2.2 Introduction

Aquaculture within the southeastern United States (Mississippi, Alabama, and Arkansas) is primarily dedicated to rearing channel catfish (*Ictalurus punctatus;* Rafinesque, 1818) and hybrid catfish (*Ictalurus punctatus*♀×*I. furcatus* (Valenciennes, 1840)♂) for food production. Aquaculture allows farmers to exert high levels of control and environmental manipulation across various production stages, which permits sustainable, high-quality, safe catfish for consumers [1]. The southeastern economy relies heavily on catfish production, with revenues reaching $398 million in sales in 2021 [2]. With competitive profit margins for large-scale production, producers have adopted more intensive aquaculture systems, such as in-pond raceways and partitioned aquaculture systems, to enhance production efficiency [3]. Though intensive production leads to increased profit yields and more efficient land use, increased stocking densities also increase the risk of disease. These risks are also exacerbated by environmental factors, such as temperature and water quality, which can increase the potential for outbreaks [4]. The catfish industry has demonstrated decades of enhancements in production methods, and fish health is a consistent target for improvement. Increased stocking densities in more intensive systems leave catfish more prone to disease, and with limited approved antibiotics or commercially available vaccines, farmers have few options for prevention and treatment [5].

Most economic losses in the catfish industry are attributed to bacterial disease, with disease-induced anorexia and direct losses from mortality events leading to decreased production [6]. Three bacterial pathogens, *Edwardsiella ictaluri, Flavobacterium covae* (formerly *F. columnare* genetic group 2)[7], and hypervirulent *Aeromonas hydrophila*,

are primarily responsible for substantial economic losses throughout the sector [8][9]. *Edwardsiella ictaluri* and *F. covae* are the causative agents of enteric septicemia of catfish (ESC) and columnaris disease, respectively, which cause significant losses on farms. Diagnostic reports from the Aquatic Research and Diagnostic Laboratory (ARDL) at Stoneville, MS, over the past decade indicate a high incidence of disease associated with *E. ictaluri* or *F. covae* [10]. These pathogens have primarily been evaluated during single infections. To better manage these disease agents, the dynamics of coinfections need to be assessed.

Generally, *E. ictaluri* has been considered a more ruinous bacterial pathogen of US farm-raised catfish [11][12]. However, columnaris disease has been a more frequent diagnosis over the past decade, accounting for 41.7% of cases submitted to ARDL compared with 32.5% for ESC from 2009 to 2019 [10]. Outbreaks of ESC typically occur when first-year fingerlings encounter the bacteria for the first time, often in late summer/early fall [13]. Infected fish exhibit lethargy, exophthalmia, cranial ulcers, ascites, and typically display abnormal swimming behaviors, including spiral swimming patterns and stargazing [14]. Lost productivity due to morbidity and mortality culminates in an estimated $60 million in annual economic losses to the industry [15].

Comparably, *F. covae* is a Gram-negative bacterium responsible for columnaris disease [7]. Columnaris disease typically presents as an external infection of the skin, fins, and gills and often in the presence of other bacterial or parasitic agents. Reports from the Louisiana Aquatic Diagnostic Laboratory in the early 1990s indicated that nearly 90% of Columnaris diagnoses were mixed infections [16]. Columnaris disease,

like ESC, is one of the leading causes of mortality in channel catfish [17], with losses due to the pathogen estimated to exceed $30 million annually [8][18].

Coinfections, which occur when a host is infected with multiple pathogens, have been reported for farm-raised catfish, although information regarding prevalence, mortality rates, and mechanisms of infection is scarce [19]. Coinfections between *E. ictaluri* and *F. covae* have been documented in catfish diagnostic cases from Alabama and Mississippi [20]. While effective treatment and prevention strategies for each pathogen have been developed [21][22][23][24][25][26], the ability of these approaches to combat coinfections is unknown, and efficacy of approved antibiotics has yet to be defined under conditions where a combination of pathogens infect a single fish.

As a first step in defining these coinfection interactions in channel catfish, these pathogens must be assessed in tandem to determine exactly how mortality is impacted along with several innate immune parameters. At present, it is unknown if dual infections of these agents interact synergistically or antagonistically in the fish host [19] and the impact of coinfections of these two agents may be underappreciated. Herein, the dynamics of *E. ictaluri* and *F. covae* coinfections were assessed in juvenile channel catfish under controlled conditions. These studies lay the foundation for future works assessing the pathophysiological and immunologic responses during mixed infections and the development of management strategies to minimize the impact these agents have on catfish health and production.

552 2.3 Materials and Methods

553 *2.3.1. Bacteria and Culture Conditions*

554 *E. ictaluri* S97-773 [27] (GenBank CP084521) was revived from cryogenic
555 storage (-80 °C) by isolation streaking onto brain heart infusion agar (BHIA) and
556 incubated for 48 h at 28 °C. Following confirmation of morphology, an individual colony
557 was expanded in 20 mL of brain heart infusion broth (BHIB; BD Biosciences; Franklin
558 Lakes, NJ, USA) for 18 h at 28 °C with shaking (175 rpm). After incubation, 100 µL of
559 broth was used to seed 250 mL of BHIB (18 h at 28 °C, 175 rpm). The final challenge
560 culture was adjusted to an optical density at 600 nm (OD_{600}) of 1.058 using sterile BHIB
561 and a Biophotometer Plus spectrophotometer (Eppendorf; Enfield, CT, USA). Similarly,
562 *F. covae* ALG-00-530 [7] (GenBank MW353001) was revived from cryostock by
563 isolation streaking on modified Shieh agar (MSA) [28] and 24 h incubation at 28 °C. A
564 single yellow-pigmented adherent rhizoid colony was subsequently transferred to a 50
565 mL conical tube containing 10 mL of sterile, modified Shieh broth (MSB) and expanded
566 for 12 h at 28 °C with shaking (175 rpm). An aliquot (5 mL) was used to seed 200 mL of
567 MSB and expanded for 12 h under the same conditions. As above, the challenge culture
568 was adjusted using sterile MSB to an OD_{550} = 0.707. Viable cell concentrations of
569 adjusted cultures were determined using standard plate count techniques and appropriate
570 media for each pathogen (*E. ictaluri*: BHIA; *F. covae*: MSB).

571

572 *2.3.2 Experimental Design (Trials A and B)*

573 Healthy, juvenile channel catfish (Marion strain; ~15 g) from the E.W. Shell
574 Fisheries Center at Auburn University (Auburn, AL) were reared in a recirculating

aquaculture system (RAS) with dechlorinated municipal water prior to study initiation. To characterize coinfections involving *E. ictaluri* and *F. covae*, catfish were arbitrarily assigned to five treatment groups (6 tanks per treatment; 20 fish per tank) for *in vivo* infectivity trials. All fish were transferred into respective tanks containing 38 L (within a 64 L tank) at 2 d prior to the challenge. The aquaria were supplied with flow-through dechlorinated municipal water at a rate of 0.5 L min^{-1} at 28 °C with supplemental aeration. Fish were both monitored and fed twice daily during acclimation. Fish were randomly distributed within tanks and treatment groups were randomly assigned to tanks, and Groups 1 and 2 were exposed by immersion to the full dose of *E. ictaluri* and *F. covae*, respectively. Group 3 received a half dose of *E. ictaluri* followed by a half dose of *F. covae* 48 h later. Conversely, Group 4 received a half dose of *F. covae* with a subsequent half dose of *E. ictaluri* after 48 h. Group 5 consisted of mock-challenged fish exposed to sterile phosphate-buffered saline (PBS; pH 7.2). The Group 5 control group received PBS twice (0 h and 48 h), just as the coinfection Groups 3 and 4 to consider any potential stress effect. Throughout the manuscript, treatments that received *F. covae* followed by *E. ictaluri* are defined as co-*F. covae*, while fish exposed to *E. ictaluri*, followed by *F. covae*, are deemed co-*E. ictaluri*. For each treatment, 3 tanks served to estimate challenge mortality, while 3 tanks were used for sampling.

During the immersion challenge, the water level was lowered to 10 L for all tanks and was restored to the normal level post-challenge. Group 1 tanks received a 6 mL inoculum (OD_{600}= 1.058) of *E. ictaluri*, bathed for 0.5 h in 10 L of rearing water (28 °C), delivering a dose of 5.4×10^5 CFU mL^{-1}. Group 2 tanks were dosed with a 110 mL inoculum (OD_{550}= 0.707) of *F. covae* for 0.5 h in 10 L, yielding a delivered dose of 3.63

598 × 10^6 CFU mL^{-1}. Group 3 tanks received 3 mL of the same *E. ictaluri* culture, delivering
599 2.7 × 10^5 CFU mL^{-1}, followed 48 h later with a 55 mL inoculum of *F. covae* culture (1.8
600 × 10^6 CFU mL^{-1}). Similarly, Group 4 tanks received 55 mL of *F. covae* inoculum
601 delivering 1.8 × 10^6 CFU mL^{-1} and subsequent 3 mL of *E. ictaluri* culture 48 h later (2.7
602 × 10^5 CFU mL^{-1}). All challenge doses were administered under the same conditions.
603 Post-initiation, tanks were monitored twice daily, and deceased fish were removed from
604 tanks. Mortality was used as the clinical endpoint for the trials. Feed was offered to fish
605 twice daily, and uneaten pellets were removed at each checkpoint. Up to 20% of daily
606 mortalities were necropsied and cultured to confirm the presence of bacteria. Coinfected
607 groups were plated on both BHIA and MSA to culture both bacteria. The end of the
608 challenge was determined once mortality had ceased for several days.

609 A second immersion trial was conducted to include additional doses equivalent to
610 those administered for coinfection treatments and to discern the contribution of half-
611 doses to mortality for each pathogen. Catfish (~22 g) were distributed to 27 tanks (3 tanks
612 per treatment; 20 fish per tank). Treatments consisted of: Full dose *E. ictaluri*, half dose
613 *E. ictaluri*, full dose *F. covae*, half dose *F. covae*, full dose *E. ictaluri* followed by full
614 dose *F. covae*, half dose *E. ictaluri* followed by half dose *F. covae*, full dose *F. covae*
615 followed by full dose *E. ictaluri*, half dose *F. covae* followed by half dose *E. ictaluri* and
616 sham challenge (sterile PBS). The *E. ictaluri* treatments received 4 mL (full) or 2 mL
617 (half) of inoculum (OD_{600}= 1.065), yielding exposure doses of 3.8 × 10^5 CFU mL^{-1} for
618 full doses and 1.9 × 10^5 CFU mL^{-1}, respectively. Similarly, the *F. covae* treatments
619 received 100 (full) or 50 mL (half) inoculums of culture (OD_{550}= 0.747) which resulted in
620 immersion baths of 7.56 × 10^6 CFU mL^{-1} for full doses and 3.78 × 10^6 CFU mL^{-1} for the

half dose. For the challenge, catfish were bathed for 0.5 h in 10 L water, and secondary doses were delivered 48 h after the initial exposure for coinfection treatments. No sampling tanks were involved in Trial B.

2.3.3. Collection and Sampling

Fish from Trial A were sampled (3 fish per tank and triplicate tanks per treatment group) at 2, 4, 7, and 21 days post-challenge. Fish were euthanized with a lethal overdose of buffered tricaine methanesulfonate (MS-222; Syndel, Ferndale, WA) at 250 mg L^{-1}. Anterior kidney, spleen, and blood were collected aseptically and used for extraction of RNA, DNA, and serological analysis, respectively. Kidney and spleen tissues were preserved in DNA/RNA Shield™ (Zymo Research Corp., Irvine, CA, USA) and stored at -20 °C until nucleic acid extraction. To assess serum lysozyme activity, fish were bled from the caudal vein using 22 ga syringes, and samples were allowed to clot overnight at 4 °C. Following separation, blood samples were concentrated at 15,000 × *g* (Eppendorf 5420; Enfield, CT, USA) for 5 min, serum collected by micropipette and stored at -80 °C until processing.

2.3.4 Bacterial DNA and tissue RNA Extraction

Reisolated bacterial colonies collected from the daily mortalities were subcultured from posterior kidneys and spleen and processed to extract DNA for endpoint PCR to confirm pathogen identity. Genomic DNA was isolated using the Omega E.Z.N.A.™ Bacterial DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA), eluted with 100 µL of provided elution buffer, quantified spectrophotometrically (Nanodrop One^{c}; Thermo

644 Fisher Scientific, Waltham, MA, USA) and stored at -20 °C until PCR analysis. Kidney
645 tissue samples, harvested at all time points, were manually homogenized in DNA/RNA
646 Shield™ (Zymo Research Corp., Irvine, CA, USA) using a mortar and pestle. RNA was
647 extracted following the Zymo Research Quick-RNA™ MiniPrep Plus kit, eluted with 100
648 µL of nuclease-free water, quantified spectrophotometrically, and stored at -80 °C.
649
650 *2.3.5. Gene expression analysis*
651 Extracted RNA was diluted to 50 ng μL^{-1} using nuclease-free water and converted
652 to cDNA using the High-Capacity cDNA Reverse Transcription Kit™ (Applied
653 Biosystems, Waltham, MA, USA) following the manufacturer's instructions. Each 20-
654 µL reaction contained 2 µL of 10x RT buffer, 0.8 µL of 25x dNTP Mix, 2 µL of 10x RT
655 random primers, 1 µL of MultiScribe™ reverse transcriptase, 500 ng of template RNA
656 and nuclease-free water to volume. cDNA was synthesized in a MiniAmp Plus thermal
657 cycler (Applied Biosystems, Carlsbad, CA, USA) programmed for one cycle of 25 °C for
658 10 min, 37 °C for 120 min, and 85 °C for 5 min and subsequently diluted to 2.5 ng μL^{-1}
659 using nuclease-free water. Four genes were evaluated for expression analysis, namely
660 *il1β, tnfa* [29], *il8* [30], and *tgfb-1* [31]. The housekeeping genes *ef1a* [30] and *actb* [32]
661 were used for normalization. The PCR was carried out in 10-µL volumes consisting of 5
662 µL PowerUp SYBR Green Master Mix™ (Applied Biosystems, Carlsbad, CA, USA),
663 forward and reverse primers at 500 nM (Supplemental Table 1), 2 µL of sample cDNA
664 nuclease-free water to volume. Each sample was run in duplicate along with no-template
665 controls consisting of nuclease-free water in place of template cDNA. PCRs were run on
666 a QuantStudio™ 5 Real-Time PCR system (Applied Biosystems Carlsbad, CA, USA)

667 programmed for initial steps of 50 °C for 2 min and 95 °C for 2 min, followed by 40
668 cycles of 95 °C for 15 sec, 58 °C for 15 sec, and 72 °C for 30 sec, with data collection
669 occurring after the 72 °C elongation. For each gene target, reaction efficiencies were
670 assessed using serial dilutions of cDNA covering five orders of magnitude, run in
671 duplicate, and starting at 10 ng. For each gene, reaction efficiencies ranging from 90-
672 110% were considered acceptable [33]. For calculations, the $2^{-\Delta\Delta Ct}$ method was
673 implemented [34], taking into consideration the combination of both housekeeping genes
674 along with the control group for each time point. Thus, each fold change of the gene of
675 interest was expressed relative to that of the control group average at that time point.
676
677 *2.3.6. Lysozyme Activity Assay*
678 Lysozyme activity was ascertained by comparisons to prepared standards
679 following previously published protocols [35]. Standards consisted of dilutions of a stock
680 480 μg mL^{-1} chicken lysozyme egg white (Rockland Immunochemicals, Pottstown, PA,
681 USA) dissolved in sodium phosphate buffer (SPB; 0.04 M Na_2HPO_4; pH 6.0) and diluted
682 to create a standard curve with a range of 0-16 μg mL^{-1}. Freeze-dried *Micrococcus*
683 *lysodeikticus* (Worthington Biochemical, Lakewood, NJ, USA) was resuspended at 0.25
684 mg mL^{-1} with SPB, and 250 μL of the bacterial suspension was added to each well, along
685 with 10 μL of sera. Each sample was run in duplicate. Absorbances at OD_{450} were
686 collected after a 20 min incubation at 37 °C with Synergy HTX Multimode Reader
687 (BioTek, Winooski, VT, USA) and compared with concurrently run standards.
688
689 *2.3.7. PCR Confirmation of Recovered Isolates*

690 The identity of presumptive *E. ictaluri* isolates recovered from dead/moribund
691 fish was confirmed by *E. ictaluri*-specific PCR. All PCR were conducted on a MiniAmp
692 thermal cycler (Applied Biosystems, Carlsbad, CA, USA). Colony PCR was performed
693 on representative colonies to confirm presumptive identification as *E. ictaluri.* Specific
694 ESC primers (ESCF and ESCR) [36] were used. Each 25-µL reaction consisted of a 12.5
695 µL 2x hot-start PCR-to-gel-master mix (Amresco LLC, Solon, OH, USA), 0.2 mM of
696 each primer, and nuclease-free water to volume. Positive (DNA extracted from *E. ictaluri*
697 (S97-773) and negative controls (nuclease-free water) were run in tandem with samples.
698 Cycle conditions were 95 °C for 5 min, followed by 30 cycles at 95 °C for 15 sec, an
699 annealing temperature of 58 °C for 15 sec, and 72 °C for 15 sec. The final extension was
700 run at 72 °C for 5 min. Aliquots of PCR products (5 µL) were separated by
701 electrophoresis through 2.0 % agarose gels in Tris-acetate-EDTA (TAE) buffer, stained
702 with GelRed (Biotium Inc., Fremont, CA, USA) and visualized by ultraviolet
703 transillumination in a Gel Doc Go imaging system (Bio-Rad, Inc., Hercules, CA, USA).
704 Samples were run alongside concurrently run molecular weight standards to confirm the
705 presence of appropriately sized bands.
706 Presumptive *F. covae* recovered from dead fish were confirmed by multiplex
707 PCR as described by [7][37]. Each 25 µL reaction contained a 12.5 µL 2x hot-start PCR-
708 to-gel-master mix (Amresco LLC, Solon, OH, USA), 2 µL of the primer cocktail (0.5 µM
709 GG-forward, 0.1 µM GG1-reverse, 0.45 µM GG2-reverse, 0.45 µM GG3-reverse, 0.3
710 µM GG4-reverse), 9.5 µL of nuclease-free water, and 1.0 µL of template
711 DNA. The cycle parameters used were: 95 °C for 5 mins, 40 cycles of 94 °C for 30
712 seconds, 56 °C for 20 secs, and 72 °C for 1 min, followed by 10 min at 72 °C. The *F.*

713 *covae* AL-02-36^{T} type strain was run as a positive control. As described above, PCR
714 products (5 µL) were resolved on a 2.0 % agarose gel via electrophoresis.
715
716 *2.3.8. Statistical Analyses*
717 Comparisons between treatment groups over time for cumulative percent mortality,
718 lysozyme activity (2, 4, and 7 DPC), and gene expression analyses (2, 4, and 7 DPC)
719 were performed using a two-way repeated measures ANOVA (α= 0.05) for treatment,
720 time, and treatment x time, with tanks included as a random factor. Serum lysozyme
721 activity was analyzed separately at 21 DPC using a one-way ANOVA due to a lack of
722 surviving fish within sampling tanks. Tukey's post hoc test was conducted when
723 treatment effects were significant (P<0.05). Statistical analysis was performed using R
724 statistical software (R core Team, 2021). All errors reported throughout the paper
725 represent the standard error of the mean among treatment tanks, as tanks were defined as
726 the experimental unit.

727 2.4. Results
728
729 *2.4.1. Infectivity Trial A*
730 Daily mortality was recorded across triplicate tanks over 21 days (Figure 1). The
731 cumulative percent mortality (CPM) of the *E. ictaluri* only group (90.0 ± 4.1 %) or the
732 two coinfection treatments (co-*E. ictaluri;* 93.3 ± 5.4 %; co-*F. covae;* 93.3 ± 2.7 %) was
733 significantly different than CPM of the *F. covae* only group (13.3 ± 5.9 %; P < 0.001),
734 indicating mortality observed in this trial was primarily due to *E. ictaluri* infection. The
735 onset of mortality in the co-*F. covae* was delayed compared with treatments receiving *E.*

ictaluri alone or first. Fish exposed to *E. ictaluri* alone or followed with *F. covae* infection first showed signs of illness such as lethargy, reduced feeding response, and exophthalmia and mortality between 4-6 days post-challenge, while fish exposed to *F. covae* followed by *E. ictaluri* challenge 48 h after *F. covae* exposure did not show signs of disease until 9 days post-challenge, although CPM of any treatment exposed to *E. ictaluri* were not significantly different ($P > 0.05$) (Figure 1).

Fish exposed solely to *E. ictaluri* exhibited exophthalmia, petechial hemorrhaging of pectoral and anal fins, internal hemorrhaging, and eye hemorrhaging (Figure 2). Fish exposed with *F. covae* presented saddleback lesions along the dorsal fin, characteristic of columnaris disease, and exhibited internal hemorrhaging of the intestines and anterior kidneys (Figure 3). Coinfected fish from both treatment combinations demonstrated a mix of single infection clinical signs exhibiting saddleback lesions and intestinal or ocular hemorrhaging (Figures 2A and 3B). Fish exposed to both bacterial pathogens contained both *E. ictaluri* and *F. covae* bacterial colonies, while bacterial colonies recovered from treatment groups exposed to only single pathogens presented only colonies from the pathogen to which they were exposed.

2.4.*2. Infectivity Trial B*

Trial B included half-doses across treatment groups and was conducted identically to Trial A (Figure 4). Treatment groups showed significant differences in CPM ($P < 0.001$). Fish exposed to *F. covae* alone averaged a CPM of 28.3 ± 11.9%. Mortality in fish administered a half-dose of *F. covae* (6.7 ± 2.7 %) was not significantly different ($P > 0.05$) compared with the full *F. covae* dose due to a high level of variability between

replicates. Mortality comparisons between full- and half-doses for each treatment group were also insignificant. The CPM (98.3 ± 1.4 %) for the full dose *E. ictaluri/ F. covae* treatment group was significantly different from the CPM of the full *F. covae* dose (28.3 ± 11.9 %; $P < 0.01$), and the half dose of *F. covae* (6.7 ± 2.7 %; $P < 0.001$). Clinical signs during Trial B were consistent with Trial A; however, fish exposed to *F. covae* and then *E. ictaluri* presented solely with saddleback lesions with mild external hemorrhaging along with distended abdomens.

2.4.*3. Serum Lysozyme Activity*

Serum lysozyme activity from Trial A was evaluated at 2, 4, and 7 days post-challenge (Figure 5). Comparisons were made amongst treatment groups at and between time points. Interactions between time and treatment were significant ($P < 0.001$). At 2 DPC, lysozyme activity was significantly elevated for groups exposed to *E. ictaluri* (co-*E. ictaluri* and *E. ictaluri*) compared with controls ($P < 0.001$). Further, the *E. ictaluri* and co- *E. ictaluri* treatment groups demonstrated significantly ($P < 0.001$) greater lysozyme activity than the co-*F. covae* and *F. covae* treatment groups. Similar results were observed at 4 DPC, although with an increase in lysozyme activity compared with 2 DPC ($P < 0.001$). Again, co-*E. ictaluri* and *E. ictaluri* treatment groups exhibited significantly greater ($P < 0.001$) lysozyme activity compared with the *F. covae* treatment and controls ($P < 0.001$). Lysozyme activity significantly increased between 2 and 4 DPC for the co-*F. covae* treatment but was not statistically different than the co-*E. ictaluri* or *E. ictaluri* treatment groups ($P > 0.05$). For all groups, lysozyme activity at 7 DPC was similar to 4 DPC, yet greater than 2 DPC ($P > 0.05$). Again, activity in the *E. ictaluri*, co-

782 *E. ictaluri*, and co-*F. covae* treatment groups were greater than the *F. covae* and control
783 ($P < 0.001$) groups. Peak lysozyme activity occurred at 4 and 7 DPC. When analyzed with
784 all other times, a significant decrease in lysozyme activity at 21 DPC was observed
785 compared with both 4 and 7 DPC ($P < 0.001$). Due to a lack of surviving fish within
786 samplings tanks, 21 DPC lysozyme activity was analyzed separately, and no significance
787 was determined between treatment groups ($P > 0.05$) (Figure 6).
788
789 *2.4.4. Gene expression analysis*
790 At 2 DPC, single *E. ictaluri* had elevated *il8* expression compared with single *F.*
791 *covae* ($P < 0.05$) (Figure 7). At 4 DPC, *E. ictaluri* and co-*E. ictaluri* treatment groups
792 exhibited greater *il8* gene expression than all other groups ($P < 0.05$). At 7 DPC, *il8*
793 expression peaked, with *E. ictaluri*, co-*E. ictaluri*, and co-*F. covae* treatment groups
794 yielding greater *il8* expression than the *F. covae* treatment and controls ($P < 0.01$). At 2
795 DPC, the *il1β* expression for *E. ictaluri* and co-*E. ictaluri* treatment groups were
796 increased compared with *F. covae* and co-*F. covae* groups as well as controls ($P < 0.01$)
797 (Figure 8). Similar levels of expression were observed at 4 DPC, although no significant
798 differences existed between treatments or controls. Similar to *il8*, there was increased *il1β*
799 expression at 7 DPC compared with 2 and 4 DPC ($P < 0.001$), with the *E. ictaluri*
800 treatment exhibiting greater *il1β* expression than the *F. covae* treatment and control
801 groups ($P<0.05$). The co-*F. covae*, and co-*E. ictaluri* treatment groups also exhibited
802 significantly greater *il1β* expression than the control group ($P < 0.01$). There were no
803 statistical differences in the expression of *tnfa* at 2 and 4 DPC (Figure 9). At 7 DPC, *tnfa*
804 was increased in the co- *E. ictaluri*, co- *F. covae*, and *E. ictaluri* treatment groups

compared with unexposed controls ($P < 0.001$). There were no significant differences in the expression of *tgfb-1* throughout the experiment (Figure 10). Interactions between time and treatment for each gene (*il1β, tgfb-1, tnfa, il8*) were evaluated and were not significant ($P > 0.05$).

2.5 Discussion

Given the prevalence of *E. ictaluri* and *F. covae* throughout US catfish aquaculture, the synergistic dynamics of these two pathogens must be evaluated to appreciate the impact of coinfections on fish health [38]. Concurrent infections are prevalent throughout aquaculture industries and occur with a variety of different pathogens [39]. Tilapia (*Oreochromis niloticus*; Linnaeus, 1758), zebrafish (*Danio rerio*; F. Hamilton, 1822), rainbow trout (*Oncorhynchus mykiss*; Walbaum, 1792), Atlantic salmon (*Salmo salar*; Linnaeus, 1758), koi (*Cyprinus rubrofuscus*; Lacépède, 1803), shrimp (suborder Caridea; Dana, 1852), and oysters (family Ostreidae; Rafinesque, 1815) all experience coinfections that can augment mortality [40][41][42][43]. Within catfish species, coinfections of *A. hydrophila* and *E. ictaluri* can increase mortality [44]. Though coinfections commonly occur, information on pathogenicity and host response is virtually unknown as most research has focused on single pathogen infections [45][46]. Additionally, this information is not frequently investigated or reported within the U.S. catfish industry. In both infectivity trials and previous coinfective work, *E. ictaluri* acts as the primary driver for mortality. In contrast, *F. covae*, though it causes mortality, acted more as a secondary pathogen within this challenge model. The co-*E. ictaluri* group did not demonstrate a difference in mortality compared with *E. ictaluri* alone, while the co-*F. covae* group displayed significantly higher mortality than the single dose of *F. covae* in

trial A, presumably due to the introduction of *E. ictaluri.* In trial B, the co-*E. ictaluri* group again exhibited higher mortality than observed for all single-infected treatment groups. Previous trials evaluating coinfections associated with *E. ictaluri* reported high mortality levels in *E. ictaluri* only groups [47]. Crumlish et al. (2010) observed high mortality (80%) caused by *E. ictaluri*, whereas *A. hydrophila* induced very low mortality (10%). Comparatively, coinfection with the two yielded 100% mortality, with much of that likely driven by *E. ictaluri* [44]. A culmination of previous coinfection trials using *E. ictaluri* and the data presented herein offers strong evidence that *E. ictaluri* is the primary pathogen under these experimental conditions. Still, it would be of interest to repeat this trial work with additional *F. covae* strains and additional pathogen implementation time points to better discern the contributions of each pathogen to the observed mortality.

In addition to assessments of cumulative mortality, there were differences in the onset of mortality, depending on which pathogen the fish were exposed to first. In the first trial, differences in the day of the first fatality appeared driven by *E. ictaluri,* with delayed fatality in fish challenged with *E. ictaluri* later. However, in the second trial, the treatment group challenged with a full dose of co-*E. ictaluri* demonstrated 98.33% CPM, while the comparable full dose of the co-*F. covae* group averaged 58.33% CPM. Although not different, this was an interesting decrease observed due to varying the timing of pathogen inoculation. This difference in onset and severity of disease between the alternation of *E. ictaluri* and *F. covae* has not previously been documented. These results suggest the possibility of potential antagonistic interactions between the pathogens depending on which pathogen fish were exposed to first. Such antagonistic interactions may cause pathogens within the host to compete for resources, thus lessening the effects

of one pathogen and causing a decrease in mortality [48]. Obtaining a complete understanding of coinfective pathogen interactions may better define the primary and secondary roles of each pathogen concerning virulence and help in developing more effective treatments and mitigation strategies, especially for species raised in natural, open pond environments [20][41]. Further histopathological assessments are needed to confirm and better characterize the clinical differences in co- versus single infections, as they are crucial in assessing disease [49].

Lysozyme within diseased channel catfish serves as one of the first immunological host defenses [50]. Within fish, lysozyme is present in the mucosal barrier and sera of fish [51]. The increases in lysozyme activity observed with coinfected channel catfish relative to single-infected fish provide further evidence that coinfections can drastically upregulate the host's innate immune response, thus giving insight into which innate immune parameters are enhanced due to infection [52]. Lysozyme activity within all groups followed the same pattern as mortality between treatment groups. While monitoring lysozyme activity between groups over 21 days, each treatment follows the same trend with lysozyme activity low at 2 DPC, increasing to maximum observed activity levels at 4 and 7 DPC, then declining at 21 DPC. The increase in lysozyme activity documented in *E. ictaluri,* co- *E. ictaluri,* and co- *F. covae* corresponds with the mortality observed within each group, suggesting that *E. ictaluri* specifically contributed to the observed mortality and increased lysozyme activity. When evaluating enzyme activity between periods (2, 4, 7, 21 DPC), 2 DPC and 21 DPC had no significant difference, coinciding with the infection's beginning and end. At 21 DPC, lysozyme activity dramatically decreased, indicating downregulation of the innate immune

components and further suggesting that surviving fish have cleared the infection. Other studies have documented lysozyme activity with *E. ictaluri* and *F. covae* during single infections. Ren et al. (2015) observed catfish exposed to *E. ictaluri* caused a substantial increase in lysozyme expression within major internal organs (liver, spleen, and kidney) and within the mucosal surface of the fish [53]. Similar results were demonstrated within these disease trials in this study. Interestingly, lysozyme activity seems to follow the expression patterns of an *E. ictaluri* infection during coinfections. In trial B, co-*F.covae* treatment had lower mortality than the co-*E. ictaluri* treatment group, but lysozyme levels appeared to increase in response to *E. ictaluri*. Monitoring lysozyme presence throughout illness provides an understanding of host immunological processes in response to specific pathogens like *E. ictaluri*. Due to lysozyme possessing antibacterial properties in the mucosa, liver, and intestinal tract [54], lysozyme activity may, to some degree, mitigate disease [55]. But in this study, lysozyme activity was correlated with fish with a severe *E. ictaluri* infection and high ultimate mortality. Thus, further experimentation would help understand the role of lysozyme (both serum and mucus) in targeting specific catfish pathogens via the host immune response.

All pro-inflammatory cytokine genes (*il8*, *tnfa*, and *il1β)* followed the same trend, where they were prominently increased leading up to 7 DPC. Concerning expression changes over time, at 2 and 4 DPC, each immune gene demonstrated no significant differences in expression, while at 7 DPC, each gene had a significant upregulation, indicating that channel catfish have an upregulation of innate pro-inflammatory genes to combat bacterial pathogens during infection. Treatment groups *E. ictaluri*, co-*E. ictaluri*, and co-*F. covae* had significantly more gene expression at day 7 than both *F. covae* and

controls. This also indicates that the higher the mortality experienced by treatment, the higher each pro-inflammatory gene is expressed. However, *tgfb-1* expression had no significant differences between the treatment groups or sampling periods, likely due to the function of *tgfb-1* as an immunosuppressive cytokine that inhibits the immune response. During infection, the immune response will upregulate genes that will aid in the fish's survival, so upregulation of *tgfb-1* would be counterproductive. To better discern the impacts of bacterial coinfection, further studies evaluating the link between pathogen and host response (i.e., transcriptomics) may allow researchers to discern the individual contribution of each pathogen on the host cytokine response. Additionally, the simultaneous influence of two bacteria may also produce exhaustion of the host metabolism, which may have a role in cytokine expression dynamics.

In summary, a major conclusion of this study was that a combined *E. ictaluri* and *F. covae* infection increased fish mortality. Within these experimental conditions, *E. ictaluri* acted as the primary driver of mortality in both trials. While *F. covae* alone resulted in low mortality, when combined with *E. ictaluri,* this pathogen caused a substantial increase in mortality. During the co- or single infection with *E. ictaluri,* an upregulation of lysozyme activity and pro-inflammatory cytokines was observed. Though several characteristics were evaluated during *E. ictaluri* and *F. covae* coinfections, future studies are needed to resolve the respective roles of each bacterial pathogen and how specific virulence factors impact host immune and other responses. A more natural coinfection disease model can aid fish health diagnosticians and channel catfish producers to better control bacterial coinfections with more rapid and accurate disease diagnostics and develop more efficient treatments that consider the presence of multiple pathogens.

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Figures

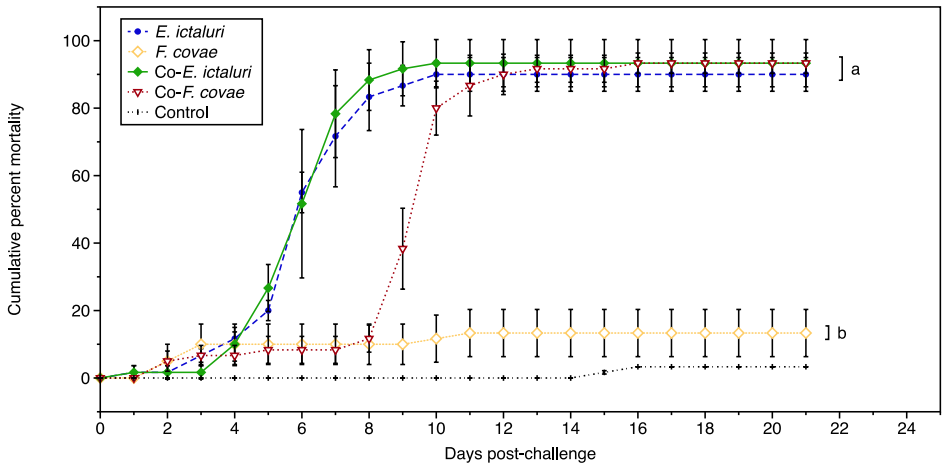

Figure 1. Cumulative percent mortality due to single infections of *E. ictaluri* and *F. covae* and co-infections from both pathogens over the entirety of the trial (21 days). Each treatment group had three tanks (n=3). Bars represent the standard error of the mean for each day.

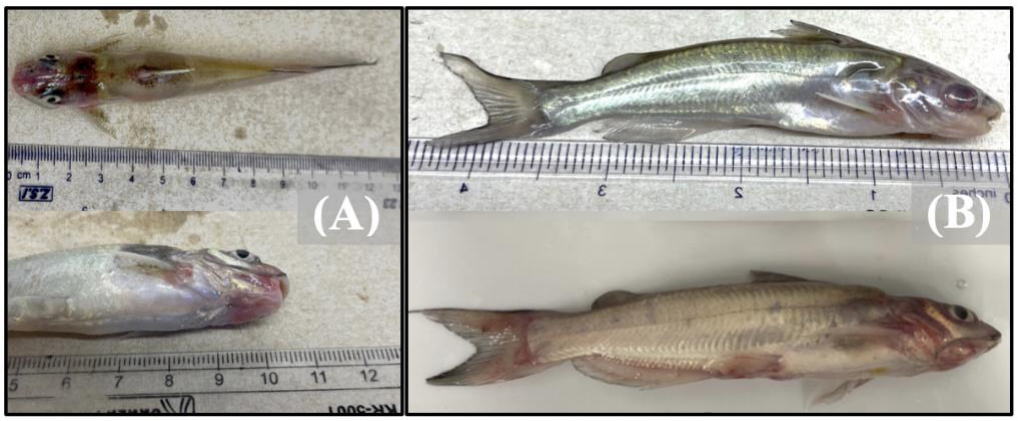

Figure 2. Images depict catfish with clinical signs due to (A) co-infection with *E. ictaluri* first, then *F. covae* 48 h post-initial inoculation, exhibiting both saddleback lesions, discoloration, and external hemorrhaging (B) infection only with *E. ictaluri*, exhibiting ocular and fin hemorrhaging and exophthalmia.

Figure 3. Images depict catfish with clinical signs due to (A) co-infection with *F. covae* first, then *E. ictaluri* 48 h post-initial inoculation, or (B) infection only with *F. covae*.

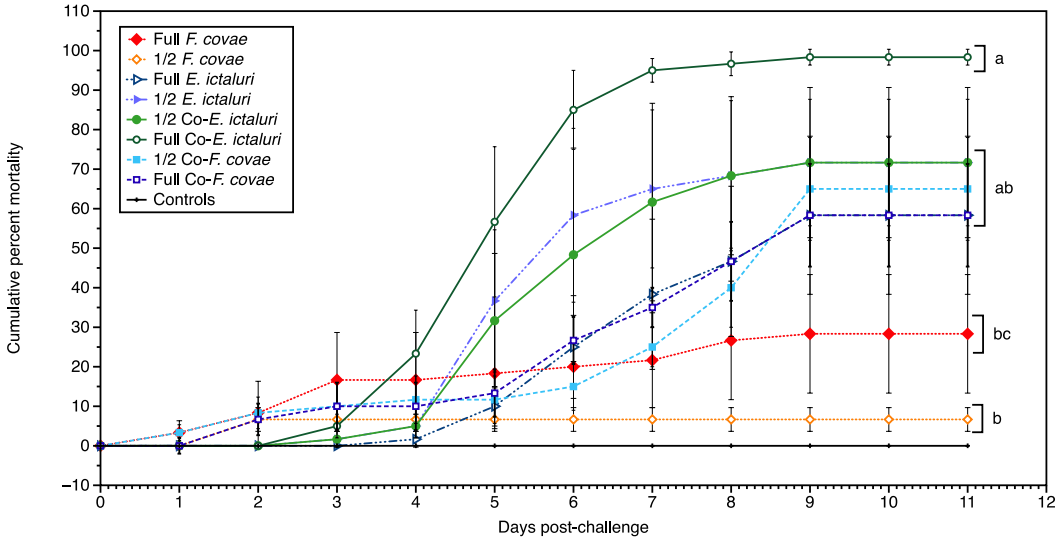

Figure 4. Cumulative percent mortality from Trial B due to single infections of *E. ictaluri* or *F. covae* and co-infections from both pathogens over the entirety of the trial (11 days). Each treatment group had three tanks (n=3). Bars represent the standard error of the mean for each day.

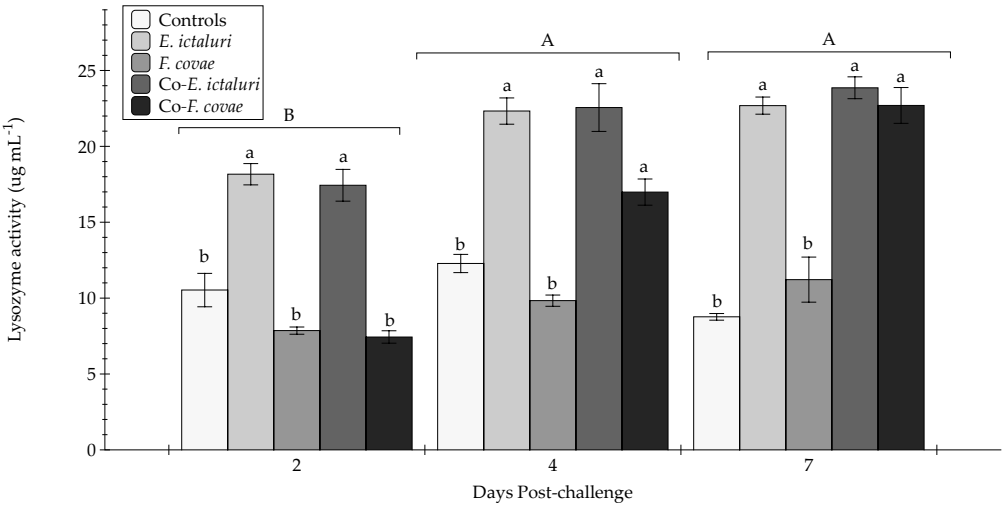

Figure 5. Lysozyme activity (µg mL^{-1}) in sera from sampled fish at 2, 4, and 7 days post-challenge. Each treatment group was conducted in triplicate (n=3). Capital letters indicate significant differences in activity between treatment time periods, and lowercase letters represent significance within treatment groups. Error bars represent the standard error of the mean for each treatment group.

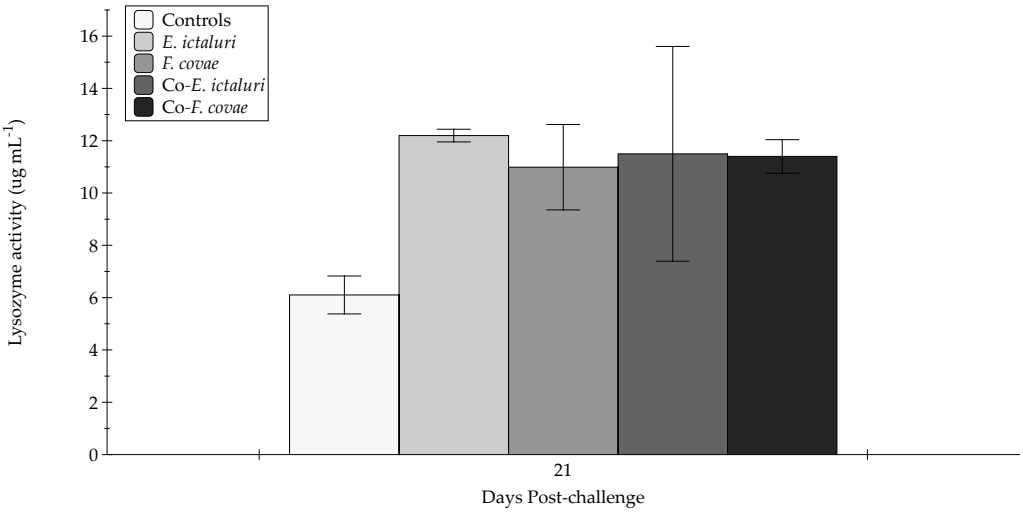

Figure 6. Lysozyme activity (µg mL^{-1}) in sera from sampled fish at 21 days post-challenge. Each treatment group was conducted in triplicate (n=3). Error bars represent the standard error of the mean for each treatment group.

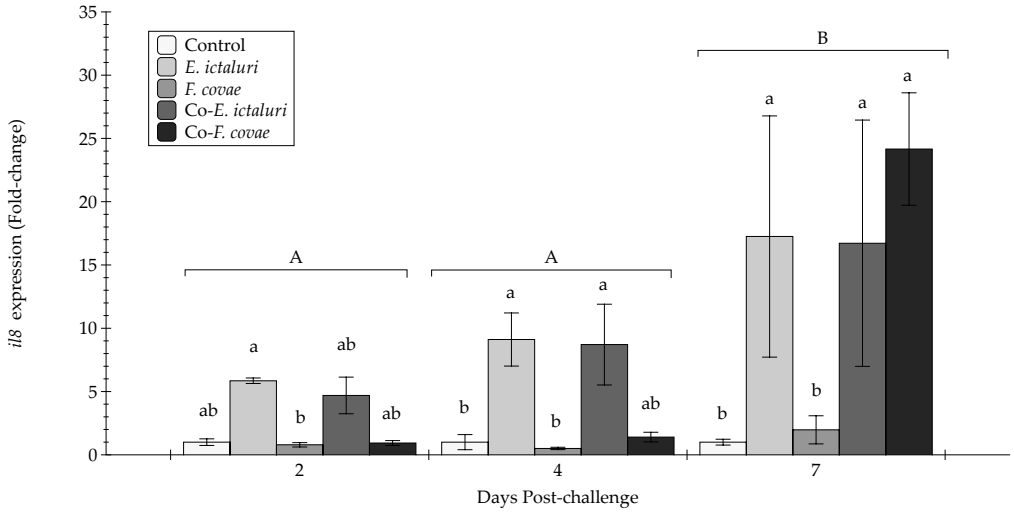

Figure 7. *il8* expression (fold-change) was evaluated from extracted anterior kidneys 2, 4, and 7 days post-challenge. Each treatment group was conducted in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Error bars represent the standard error of the mean for each treatment.

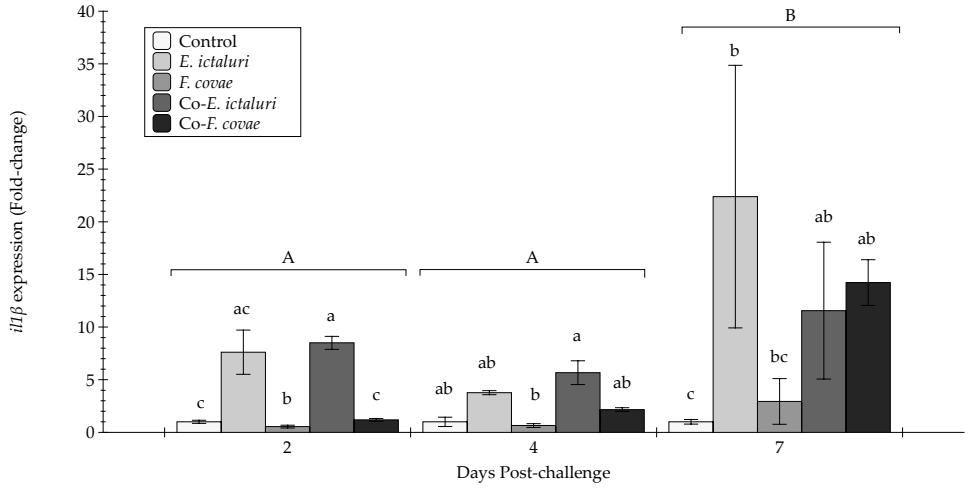

Figure 8. *il1β* expression (fold-change) was evaluated from extracted anterior kidneys during 2, 4, and 7 days post-challenge. Each treatment group was conducted in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Bars represent the standard error of the mean for each treatment.

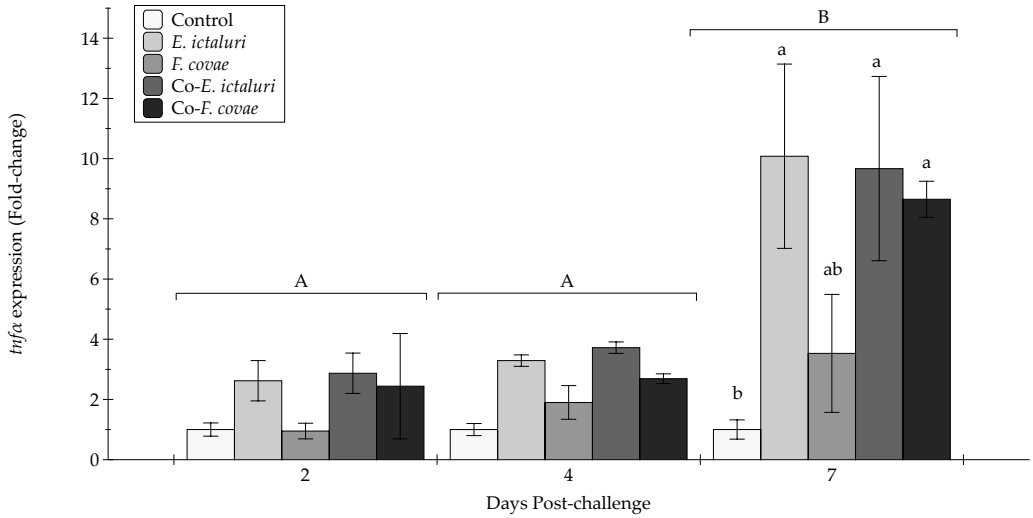

Figure 9. *tnfa* expression (fold-change) was evaluated at 2, 4, and 7 days post-challenge. Each treatment group was conducted in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Bars represent the standard error of the mean for each treatment.

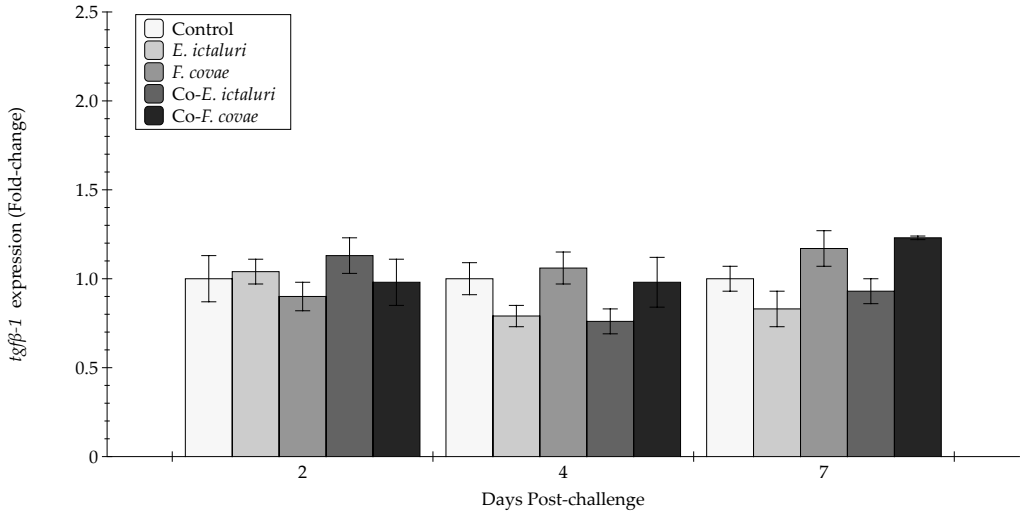

Figure 10. *tgfb-1* expression (fold-change) was evaluated from extracted anterior kidneys during 2, 4, and 7 days post-challenge. Each treatment group was assessed in triplicate (n=3). Bars represent the standard error of the mean for each treatment.

Chapter 3:

Coinfection of Channel Catfish (*Ictalurus punctatus*) with Virulent *Aeromonas hydrophila* and *Flavobacterium covae* Exacerbates Mortality

3.1 Abstract

Flavobacterium covae and virulent *Aeromonas hydrophila* are prevalent bacterial pathogens within the U.S. catfish industry that can cause high mortality in production ponds. An assessment of *in vivo* bacterial coinfection with virulent *A. hydrophila* (ML09-119) and *F. covae* (ALG-00-530) was conducted in juvenile channel catfish (*Ictalurus punctatus*). For the pathogen challenge, catfish were divided into seven treatments: 1) mock control; 2 and 3) full and half doses of virulent *A. hydrophila*; 4 and 5) full and half doses of *F. covae*; 6 and 7) coinfection full and half doses of virulent *A. hydrophila* and *F. covae*. In addition to the mortality assessment, head kidney and spleen tissues were collected to evaluate immune gene expression and quantify bacterial load using qPCR. At 96 h post-challenge (hpc), the full-dose, single virulent *A. hydrophila* infection (immersed in 2.3×10^7 CFU mL^{-1}) resulted in final cumulative percent mortality (CPM) of 28.3 ± 9.5 %. The CPM for the full dose *F. covae* group (immersed in 5.2×10^6 CFU mL^{-1}) was 23.3 ± 12.9 %. When the single pathogens were compared to the coinfections, the coinfective full-dose combination (98.3 ± 1.36) and half-dose combination (76.7 ± 17.05 %) significantly increased mortality ($P < 0.001$). Sera lysozyme activity among treatment groups was not different, yet a significant increase ($P<0.002$) in lysozyme activity was observed at 12 hpc, and a decrease was observed at 96 hpc ($P<0.001$). Three proinflammatory cytokines (*tnfa, il8, il1b* demonstrated increased expression at 48 hpc. The results emphasize the importance of evaluating coinfections and demonstrate dramatic increases in mortality when two pathogens are combined, even at half-doses. The synthesis of these mortality and health metrics will aid fish health diagnosticians and

channel catfish producers in developing therapeutants and prevention methods to control bacterial coinfections better.

3.2 Introduction

Aquaculture in the United States provides sustainable fish stocks and is a rapidly growing industry [1]. The southern economy tremendously depends on aquaculture revenue from farming catfish [2], and catfish production alone in 2021 accounted for $398 million in sales [3](NASS 2022). Aquaculture practices have improved, allowing more efficient husbandry techniques yielding higher-quality catfish [4]. Along with enhanced rearing techniques, the intensity at which the fish are being produced has also been adapted. Nevertheless, increased production can potentiate risks for disease outbreaks [5,6].

Bacterial diseases are the leading cause of losses within the catfish industry. Not only do disease outbreaks decimate potential fish stocks, but the medicated feed used to treat surviving fish also comes at a high cost to farmers, with the potential for antimicrobial resistance within prevalent pathogens [7,8]. Due to the cost of the already limited selection of available antimicrobial treatments, disease prevention within all aquaculture industries is essential to avoid significant economic losses [9,10]. Though several factors contribute to losses within the industry, *Flavobacterium covae* (formerly *F. columnare* genetic group 2) and virulent *Aeromonas hydrophila* (vAh) significantly contribute to the majority of losses within this sector and are opportunistic pathogens [11–13].

F. covae alone can contribute upwards of $30 million in annual losses and can lead to 90% mortality within production ponds during severe infections [14]. *F. covae* affects channel catfish by causing columnaris disease, which primarily manifests as an external infection but may also be systemic [15]. The pathogen first interacts with the host externally by penetrating the gills, leading to the asphyxiation of the host [16]. Clinical signs associated with columnaris include gill, skin necrosis, and frequently a distinct dorsal lesion known as saddleback [17]. There are few treatments and preventative strategies to mitigate *F. covae* infections, ranging from antimicrobial feed to vaccination. *F. covae* vaccines have demonstrated variable efficacy and are not a reliable treatment, but they are a promising disease prevention avenue [18].

Aeromonas hydrophila, a gram-negative bacterium, is responsible for motile aeromonad septicemia (MAS) and infects many fish hosts. *A. hydrophila* is typically an opportunistic pathogen and can cause devasting effects when coupled with stressors brought onto the fish [19]. Fish afflicted with the disease demonstrated multiple clinical signs, including severe internal and external hemorrhaging throughout the fish. The first mass mortality outbreak of MAS resulting from a virulent strain of *A. hydrophila* occurred in 2009 [12]. This outbreak caused high mortalities and immense economic losses ($35 million annually). Though *A. hydrophila* is often opportunistic, it is unknown if vAh, which typically causes intense mortality over a short period, acts as the primary pathogen or requires initial stressors before infection [20,21].

Reports of coinfections associated with *F. covae* and vAh are problematic due to the lack of information on how either pathogen manifests within the host during dual infections and with the potential to cause massive amounts of mortality. Coinfections

exist within multiple aquaculture industries affecting many hosts with any combination of pathogens [22–24]. Though coinfections are well-known in the catfish industry, information is scarce due to a lack of knowledge of prevalence due to underreporting and the mechanism associated with outbreaks [25]. Documented diagnostic cases in the southeastern region of the U.S. have reported coinfections between the two pathogens [13]. However, with respect to treatment, antibiotic efficacy during coinfections remains unclear. Treatments approved for single infections have not been efficiently tested during coinfections. In addition to few available treatment options, infections involving *F. covae* may increase the likelihood of coinfections with other opportunistic pathogens by creating a portal of entry through lesion lesions and damaged gill tissue [11,12, 26]. Coinfective interactions and their impact on mortality and potential to increase already catastrophic losses are unknown. Assessing mortality and immune responses through an experimental challenge model provides crucial information on the severity and a preliminary understanding of immunological effects. Due to the severity of mortality each pathogen elicits, we hypothesized that coinfection of vAh and *F. covae* would increase mortality in coinfected treatment groups. Therefore, this study documents the coinfective effects of vAh and *F. covae* on mortality and the associated immune response in channel catfish. The investigation of these parameters will shed light on improved mitigation strategies and future treatment approaches for these complex bacterial interactions.

3.3 Materials and Methods

3.3.1 Bacteria and Culture Conditions

A pure culture of vAh (ML09-119; [27,28]) was recovered from cryostock on tryptic soy agar (TSA) at 28 °C for 24 h. A single colony pick was expanded in 20 mL of tryptic soy broth (TSB) containing 0.4 mM xenosiderophore (iron chelator) deferoxamine mesylate (DFO; Sigma, St. Louis, MO, USA; [29]), and the culture was incubated at 28 °C for 12 h, 175 rotations per minute (rpm). After incubation, 20 mL of broth was used to inoculate 1 L of TSB with 0.4 mM of DFO (12 h at 28 °C, 175 rpm). The final inoculation broth was adjusted to OD_{600}=2.026 using a Biophotometer Plus spectrophotometer (Eppendorf; Enfield, CT). *Flavobacterium covae* (ALG-00-530; [11,30]); was also revived from cryostock and grown on modified Shieh agar (MSA; [31] for 24 h at 28 °C. A single colony was subsequently transferred to a 50 mL conical tube containing 12 mL of sterile, modified Shieh broth (MSB) and expanded for 12 h at 28 °C with shaking (175 rpm). An aliquot (6 mL) was used to seed 200 mL of MSB and expanded for 12 h under the same conditions. As mentioned above, the challenge culture was adjusted using sterile MSB to an OD_{550} = 0.707. Colony forming units (CFU) mL^{-1} of the adjusted cultures were determined using standard spread plate count techniques and appropriate media for each pathogen (virulent *A. hydrophila*: TSA; *F. covae*: MSA).

3.3.2 Experimental Design

F. covae and vAh coinfections were characterized using seven treatment groups in triplicate tanks (20 fish $tank^{-1}$) during an *in vivo* pathogen challenge with ~22 g channel catfish (Marion strain; Table 1). Groups 1 and 2 were challenged by immersion with full and half doses of *A. hydrophila*, while groups 3 and 4 were challenged with half doses

and full doses of *F. covae*. Groups 5 and 6 were challenged by immersion with half and full doses of each pathogen (coinfection groups). Group 7 consisted of mock-challenged fish, and fish were exposed to sterile phosphate-buffered saline (PBS; pH 7.2) in lieu of bacterial inoculum (Figure 11). Supplementary sampling tanks (n = 21, 3 tanks per group) were used to replicate each treatment group to collect timepoint tissues without disturbing mortality data. These sampling tanks were subjected to the same inoculum and pathogen challenge conditions.

3.3.3 Immersion Challenge

Prior to the challenge, all fish were transferred and randomly allocated into respective 60 L tanks containing 37.9 L. Catfish were acclimated for 2 d, in which they were monitored and fed twice daily a commercial floating catfish diet (Optimal Fish Food, Omaha, Nebraska). Tanks were supplied with 28°C ± 1°C dechlorinated municipal water at a rate of 0.4 L min^{-1}, and supplemental aeration was provided via an airstone. Dissolved oxygen (DO) was maintained level between 8.0 ± 1.0 mg L^{-1}. All channel catfish, including controls, were fed to satiety 4 h before the challenge. The adipose fin was clipped following anesthetization in water containing 100 mg L^{-1} of tricaine methanesulfonate (MS-222; Syndel, Ferndale, Washington) and 100 mg L^{-1} of sodium bicarbonate [32]. Then, the water flow to each tank was turned off, the water level was lowered to 10 L, and the volume of bacteria or PBS for the mock challenge was added. Fish were exposed for 1 hour, and the water flow was restored to 0.5 L min^{-1} [29]. Single pathogen groups were inoculated with 100 mL for full doses and 50 mL for half doses of the respective pathogen. Coinfected groups received 100 mL (full) and 50 mL (half) of

both bacteria. Groups exposed to vAh (half and full doses of single vAh and coinfected treatment groups) received 2.3×10^7 CFU mL^{-1} at full dose and 1.1×10^7 CFU mL^{-1} at half doses. Fish exposed to *F. covae* (half and full doses of single *F. covae* and coinfected treatment groups) were inoculated with 5.2×10^6 CFU mL^{-1} (full doses) and 2.6×10^6 CFU mL^{-1} (half doses) (Table 3).

3.3.4 Collection and Sampling

Fish were sampled in triplicate (n=3 tanks, with 3 fish per tank) from each group at 6 h, 12 h, 24 h, 48 h, and 96 hours post-challenge from supplementary sampling tanks. Anterior kidney, spleen, and blood were collected aseptically and used to extract RNA, DNA, and sera, respectively. Kidney and spleen tissues were preserved in DNA/RNA Shield (Zymo Research Corp., Irvine, California) and stored at -20 °C until nucleic acid extraction. Sera for lysozyme activity was collected by bleeding fish from the caudal vein using 22-gauge syringes and placing blood into microtubes. Blood was allowed to clot overnight at 4 °C, centrifuged at 16,000 × *g* (Eppendorf 5420; Enfield, CT) for 5 min, and then sera were harvested and stored at -80 °C until needed. Post-challenge, only deceased fish were removed from tanks. Kidney and spleen tissue from 20% of daily mortalities were cultured to confirm the cause of death. Tissues from coinfected groups were plated on TSA and MSA to target both bacteria.

3.3.5 DNA and RNA Extraction

Spleen tissues were homogenized with pestles within 1.5 mL microtubes. According to the manufacturer's directions, DNA was extracted using the Omega

E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Inc., Norcross, Georgia). Kidney tissue samples were manually homogenized with pestles in DNA/RNA Shield (Zymo Research Corp., Irvine, California). RNA was extracted using a Zymo Research Quick-RNA™ MiniPrep Plus kit (Zymo Research Corp., Irvine, California) according to the manufacturer's directions. Reisolated *F. covae* bacterial pellets collected from fresh mortalities were processed to extract DNA for endpoint PCR to confirm pathogen identity. Genomic DNA was isolated using the Omega E.Z.N.A.® DNA Kit (Omega Bio-tek, Inc., Norcross, Georgia, USA) according to the manufacturer's directions. Virulent *A. hydrophila* isolates were confirmed through colony pick PCR using a vAh-specific primer set. All DNA and RNA samples were eluted with 100 µL of nuclease-free water. Extracted RNA and DNA samples were quantified spectrophotometrically with Nanodrop One^{c} (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stored at -20 °C until needed.

3.3.6 Gene expression analysis

Extracted RNA was diluted to 50 ng μL^{-1} using nuclease-free water and converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA), according to assay directions. Each 20 µL reaction contained 2 µL of 10x R.T. buffer, 0.8 uL of 25x dNTP Mix, 2 µL of 10x R.T. random primers, 1µL of Multiscribe™ reverse transcriptase, 500 ng of template RNA and nuclease-free water to volume. cDNA samples were synthesized in a MiniAmp Plus thermal cycler (Applied Biosystems, Carlsbad, California) programmed for a single cycle of 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Once the reaction was

complete, the cDNA-was diluted to 2.5 ng μL^{-1} with nuclease-free water. Four genes were evaluated for expression analysis, namely *il-1β, tnfα* [33], *il8* [34], and *tgfβ–1* [35]. The housekeeping genes *ef1α* [36] and *actb* [24] were used for normalization. The PCR was performed in 10-μL volumes consisting of 5 μL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 0.25 μM of each forward and reverse primer (Supplemental Table 1), 3 μL of sample cDNA nuclease-free water to volume (1 μL). Each sample was run in duplicate along with no-template controls consisting of nuclease-free water in place of template cDNA. qPCR was run on a QuantStudio™ 5 Real-Time PCR system (Applied Biosystems) programmed for initial steps of 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec, 58 °C for 15 sec, and 72 °C for 30 sec, with data collection occurring after the 72 °C elongation. For each gene target, reaction efficiencies were assessed using serial dilutions of cDNA covering five orders of magnitude, run in duplicate, and starting at 50 μg. For each gene, reaction efficiencies ranging from 90-110% were considered acceptable [37]. The $2^{-\Delta\Delta Ct}$ method was used to calculate all gene expression values [38], combining housekeeping genes (*ef1α* and *actb*) and control groups for each sampling period, allowing each fold change of each gene to be expressed relative to control averages.

3.3.7 Lysozyme Activity Assay

Lysozyme activity was quantified following previously published protocols [39]. Lysozyme standards consisted of serial dilutions of chicken lysozyme egg white (Rockland Immunochemicals, Pottstown, Pennsylvania, USA) dissolved in sodium phosphate buffer (SPB; 0.04 M Na_2HPO_4; pH 6.0), and diluted to create a standard curve

with a range of 0-16 µg mL-1. Freeze-dried *Micrococcus lysodeikticus* (Worthington Biochemical, Lakewood, New Jersey, USA) was resuspended into 40 mL SPB at 0.25 mg $\mu L^{-1,}$ and 250 µL of the bacterial suspension added to each well, along with 10 µL of sera. Each sample was run in duplicate. Absorbances at OD_{450} were collected after a 20 min incubation at 37 °C with a Synergy HTX™ Multimode Reader (BioTek, Winooski, Vermont, USA) and compared to a standard curve assembled from prepared standards mentioned above run in tandem.

3.3.8 Quantification of Bacterial Load

Quantitative polymerase chain reaction (qPCR) was performed with QuantStudio 5 Real-Time PCR instrument (Applied Biosystems, Carlsbad, California) to quantify the bacterial load within sampled spleens. Extracted DNA samples were diluted to 10 ng μL^{-1}. To quantify vAh present in splenic tissue, each qPCR reaction consisted of 9.5 µL of TaqMan Fast Advanced Master Mix (Applied Biosystems, Carlsbad, California), 1 µL of forward and reverse primers (20 mM; S1), 1 µL of vAh probe (2 mM) (Supplementary Table 1; [40]), 1 µL of spud template DNA (500 copies), 0.5 µL of SPUD primers (Supplementary Table 1), 0.5 µL of SPUD probe (Supplementary Table 1; [41]), 5 µL of template (50 ng $reaction^{-1}$) and 6.5 µL of nuclease-free water. Run conditions were programmed with a denaturation for 15 min at 95 °C, 40 cycles of another 15 sec denaturation at 95 °C, and an annealing step at 60 °C for 1 min. The data collection occurred at the end of the annealing step of each cycle [40,42]. *F. covae* was quantified in spleen tissue by running the same reaction with *F. covae*-specific primers and probe (Supplementary Table 1; [42]). Primer concentrations and run conditions were identical

for quantifying *vAh* and *F. covae.* SPUD DNA and probes were a positive control to ensure no inhibition was present. Each reaction plate contained samples run in duplicate along with negative controls (no template, only TE buffer) and five serially-diluted standards of the target bacteria [43].

3.3.9 PCR Confirmation

The presence of each bacterial pathogen in single and coinfected treatment groups was evaluated from challenge mortalities. All PCR reactions were conducted on a MiniAmp thermal cycler (Applied Biosystems, Carlsbad, CA). Colony PCR was performed on representative colonies to confirm presumptive identification as *vAh* by harvesting a single bacterium colony from a TSA plate and inserting it directly into the following reaction. Specific *vAh* primers (2968F and 2968R;[44]) were incorporated into the assay. Each 25 μL reaction consisted of a 12.5 μL hot-start master mix (Trilink BioTechnologies, San Diego, CA), 0.5 μL 2968F (10 μM stock), 0.5 μL (10 μM stock) of 2968R, and nuclease-free water to volume. Positive (DNA extracted from vAh (ML09-119)) and negative (nuclease-free water) controls were run in tandem with samples. Cycle conditions were 95°C for 5 min, followed by 30 cycles at 95°C for 15 sec, an annealing temperature of 58°C for 15 sec, and 72 °C for 15 sec. The final extension was run at 72°C for 5 min. Aliquots of PCR products (5 μL) were separated by electrophoresis through 2.0% agarose gels in Tris-acetate-EDTA (TAE) buffer, stained with GelRed (Biotium Inc., Fremont, California, USA) and visualized by ultraviolet transillumination in a Gel Doc Go imaging system (Bio-Rad, Inc., Hercules, California,

USA). Samples were run alongside concurrently run molecular weight standards to confirm the presence of appropriately sized bands (200 bp).

DNA extracted from presumptive *F. covae* colonies recovered from dead/moribund fish was confirmed by multiplex PCR [11,45]. Each 25 µL reaction contained a 12.5 µL hot-start master mix (Trilink BioTechnologies, San Diego, CA), 2 µL of a primer mixture (0.5 µM GG-forward, 0.1 µM GG1-reverse, 0.45 µM GG2-reverse, 0.45 µM GG3-reverse, 0.3 µM GG4-reverse), 9.5 µL of nuclease-free water, and 1.0 µL of gDNA (5 ng μL^{-1}). The cycle parameters used were: 95 °C for 5 mins, 40 cycles of 94 °C for 30 seconds, 56 °C for 20 secs, and 72 °C for 1 min, followed by 10 mins at 72 °C. The *F. covae* AL-02-36^T type strain was run as a positive control. As described above, PCR products (5 µL) were also resolved on a 2.0 % agarose gel via electrophoresis and confirmed with a positive control (300 bp).

3.3.10 Statistical Analyses

Comparisons between treatment groups and time for cumulative percent mortality, lysozyme activity, and gene expression analyses were performed using repeated measures two-way ANOVA (a = 0.05) evaluating treatment, time, treatment × time, and incorporating tanks as a random factor. Data from 48 and 96 hpc was not included for the full dose coinfection treatment due to a lack of surviving fish (tanks had reached 100% mortality in sampling tanks). Tukey's post hoc test was incorporated when differences were significant ($P<0.05$) to identify which groups were different. Statistical analysis was conducted using R statistical software (R core Team, 2021). All errors reported represent the standard error of the mean between treatments.

3.4 Results

3.4.1 Mortality due to in vivo pathogen challenge

Mortality was recorded daily throughout the trial, and endpoint cumulative percent mortality (CPM) was calculated (96 h; Figures 12). Treatment groups exposed to vAh (half and full vAh and coinfected groups) began dying at 6 h. Full and half vAh doses stopped experiencing mortality at approximately 48 h, while full dose coinfected groups stopped dying 12 h prior. Mortality for full and half *F. covae* treatment groups only began between 24-48 h and ceased at 84 h. All single infection groups at both full and half doses (*F. covae* and vAh) had significantly lower CPM than coinfected groups (half and full doses) ($P < 0.05$). The full-dose coinfected group experienced 98.3 ± 0.3 % CPM; even at half doses of both pathogens, the coinfected group still showed high mortality (76.7 ± 4.2 %). Full doses of single *F. covae* (23.3 ± 2.6 %) and vAh (28.33 ± 1.5%) did not reach half of the mortality observed in half or full-dose coinfected groups. Half doses of single-infected *F. covae* (10.0 ± 1.0 %) and vAh (23.3 ± 1.9 %) groups were not significantly less than the respected full doses for each group ($P > 0.05$). All control tanks experienced no mortality throughout the trial.

3.4.2 Presence of Clinical Signs

Fish exposed to vAh exhibited severe ocular hemorrhaging and external hemorrhaging of the anal and caudal fins (Figure 18). Internally, fish demonstrated hemorrhaging within the gastrointestinal tract. Clinical signs due to *F. covae* were typical of this infection, with discoloration, gill necrosis, and dorsal lesions (saddleback). Fish

coinfected with both pathogens demonstrated an amalgam of clinical signs. Clinical signs also differed depending upon the disease stage over time. Deceased fish collected soon after the initial infection (12 h) either had discoloration consistent with *F. covae* infections or only mild external hemorrhaging (typical of *vAh* infection) and internal hemorrhaging (Figure 19). In comparison, mortalities at 36 h exhibited extreme external and internal hemorrhaging and epithelial sloughing consistent with clinical symptoms from both pathogens (Figure 20).

3.4.3 Sera lysozyme activity

Sera lysozyme activity was compared among all treatment groups (half and full doses of coinfected and single infected groups) at 6, 12, 24, 48, and 96 hpc (Figure 13). A significant interaction ($P < 0.05$) was present between time and treatment. Comparisons between time demonstrated increased activity at 12 hpc compared to 6, 24, and 48 hpc ($P < 0.05$). However, a significant decrease in activity was observed at 96 hpc compared to all other periods ($P < 0.05$). There were no significant differences between treatment groups at 6, 12, 24, and 96 hpc ($P > 0.05$), while at 48 hpc, treatment differences were observed ($P < 0.05$). Catfish challenged with full doses of vAh, and *F. covae* exhibited significantly higher lysozyme activity at 48 hpc than the control group ($P < 0.05$). The half vAh group, when compared to full *F. covae* treatment, had significantly lower lysozyme activity ($P < 0.05$).

3.4.4 Gene expression analysis

The expression of *il1ß* gene was evaluated at 6, 12, 24, 48, and 96 hpc (Figure 14). An increase in expression was observed at 48 hpc compared to all other time points ($P < 0.05$); however, there were no significant differences between treatment groups at all sampling times ($P > 0.05$). Similar expression patterns were observed with the *il8* gene, except no significant differences were observed between 96 and 48 hpc ($P > 0.05$; Figure 15). The expression of *tgfb* and *tnfa* genes were evaluated, and differences were only detected between periods for both genes (Figures 16 and 17). An increase in *tgfb* expression was seen at 48 hpc compared to 12 hpc ($P < 0.05$). No significant interactions were seen between time and treatment for all genes evaluated ($P < 0.05$). All three proinflammatory cytokines demonstrated an increase in expression at 48 hpc.

3.4.5 Quantification of bacterial load

The bacterial load of each pathogen was quantified at 6, 12, 24, 48, and 96 hpc (Figure 21). For vAh, the bacteria were present in all groups exposed to vAh at 6 h. At 12 hpc, only full dose vAh and full dose coinfected groups displayed the presence of virulent *A. hydrophila* in the splenic tissue. The coinfected treatment receiving a full dose of both pathogens was the only group with detectable vAh copies at 24 hpc. At 48 and 96 hpc, no bacteria were detected in any treatments. Unfortunately, full-dose, coinfected catfish at 48 and 96 h could not be measured due to a lack of surviving fish. No significant differences were seen between time or within treatment groups ($P > 0.05$).

The bacterial load quantified for *F. covae* increased at 6 hpc compared to 12 and 48 hpc ($P < 0.05$). Only at 6 and 48 hpc, were differences among treatment groups observed. At 6 hours, full coinfection was statistically different from controls and full *F. covae* ($P <$

0.05). Both of which had no detectable presence of *F. covae*. However, at 48 h, full *F. covae* had significantly more bacteria present than all other treatments ($P < 0.05$).

3.5 Discussion

Coinfections occur in multiple aquaculture industries, causing significant mortality and economic losses [46–48]. Coinfections amongst opportunistic pathogens have been seen to augment mortality severity during challenges, thus increasing the potential economic losses in production ponds [49]. Within the catfish industry, coinfections have become an increasing threat amongst vAh and *F. covae* due to the increased prevalence of *F. covae* in catfish production and the ability to act as a portal of entry for the opportunistic vAh [13]. Though the threat of coinfection is highly probable and lethal in production ponds, little is known concerning infection mechanics, severity, and mitigation strategies [50]. This lack of information is supplemented due to the majority of research focusing on the effect of single infections [17], thus presenting a great need for research into coinfective bacterial pathogens within the catfish industry [51]. This study evaluated the effects of two opportunistic pathogens (vAh and *F. covae*) on mortality when channel catfish are infected concurrently. Previous studies have observed coinfections associated with these two bacterial pathogens investigating each but in combination with another pathogen within a different host [49,52].

Channel catfish exposed to both pathogens experienced severely augmented mortality at half the dose compared to full single infection doses. Providing more evidence of how coinfections can impact production ponds, thus shedding light on how crucial it is to assess coinfections amongst other devastating pathogens. The interactions

observed here detailed how portals of entry created by pathogens increase the likelihood of a coinfection, thus increasing mortality. This presents a possible explanation for why a coinfection with a bacterium creating a portal of entry (*F. covae*) and a bacterium that relies on internal exposure (vAh) create such a deadly combination.

Lysozyme is a primary immunological barrier, protecting hosts from potential pathogens [53]. Lysozyme is present throughout the mucosa of channel catfish and is contained within their sera [54]. Lysozyme activity was investigated in this study to measure one of the host's innate immune responses due to coinfections. The present study detected few differences in lysozyme activity among treatment groups. This contrasted with a study by Wise et al. (2023) which documented that coinfected groups exposed to *E. ictaluri* and *F. covae* experienced significantly more lysozyme activity than single-infected groups. Authors noted that *E. ictaluri* acted as the primary driver for mortality and lysozyme activity [55]. However, a coinfection between two opportunistic pathogens (*F. covae* and vAh) presented no differences except at 48 hpc. Single full-dose infections were the only two treatment groups that experienced slightly elevated activity compared to controls. For *Aeromonas* spp. infections, it has been previously documented that *A. veronii* infection increased lysozyme expression in grass carp (*Ctenopharyngodon idellus*; [56]) and that *A. hydrophila* increased lysozyme activity for up to 21d in blunt nose sea bream (*Megalobrama amblycephala*; [57]). Unfortunately, samples from coinfected treatment groups were not obtainable at 48 or 96 h due to total fish mortality at full doses. At half doses, coinfected groups did not present a difference in activity to other treatments. Though there were no differences between treatments, there were slight differences between time. Valuable information about the onset and lapse of infection

1819 was provided due to the increase observed at 12 hpc compared to 6 h, 24 h, and 48 h,
1820 with a decrease at 96 hpc. The decline at 96 hpc in lysozyme activity, combined with the
1821 lack of mortality, was indicative of the end of the infection. While the initiation of
1822 infection occurred between 6 and 12 hpc, coinciding with the mortality. Little research
1823 has evaluated sera lysozyme activity within channel catfish exposed to vAh. Previous
1824 work investigated *A. hydrophila*'s effects on lysozyme activity but within other hosts [57]
1825 These results provide insight into novel infection dynamics (beginning, peak, and end of
1826 infection) of both single and coinfections between *F. covae* and vAh. Simultaneously,
1827 illustrating *F. covae* and vAh coinfections do not elicit a significant increase in lysozyme
1828 activity compared to single infections.

1829 Bacterial load was measured for each bacterium within the spleen. Results
1830 indicated higher doses persist longer within host spleens than half doses regardless of a
1831 coinfection at 12 hpc for vAh. A trend was observed depicting a high presence vAh early
1832 in the infection (6 hpc), then no presence of 48 or 96 hpc. Similar results were seen in
1833 previous studies. Zhang et al. (2016) conducted a waterborne vAh and measured bacterial
1834 load within internal tissues. This research observed the highest quantities of vAh within
1835 the spleen being between 1 to 4 hpc, and no vAh was detected at 48 hpc [58]. Both
1836 studies depict an increase in vAh presence within the spleen early in infection and no
1837 presence after 48 hpc. This corresponds with mortality trends observed in this study. Fish
1838 began to experience death between 6-12 hpc during the vAh infection, which correlates
1839 with the bacterial presence detected within internal organs.

1840 Proinflammatory cytokines (IL-8, TNF-α, and IL-1β) and an immunosuppressive
1841 cytokine (TGF-β) were measured to evaluate the innate immune system during

coinfections. Interestingly, all genes demonstrated significantly higher expression at 48 hpc , primarily induced by vAh, but no treatment differences were observed within any genes. These results present critical information about coinfection immune responses. These cytokines levels were not affected during coinfection despite severely exponentiated mortality. However, differences in expression between single and coinfected treatment groups could not be made due to sample variation.

In summary, a coinfection between *F. covae* and virulent *A. hydrophila* under these laboratory conditions significantly increases mortality compared to single infections of both pathogens. Even half coinfective doses caused more mortality than full doses of single pathogens. Previous studies have characterized both *A. hydrophila* and *F. covae* as more opportunistic, causing devastating mortality when fish are stressed. This study demonstrates that combining two ubiquitous pathogens can significantly affect mortality, thus potentially augmenting the economic impact. Along with critical mortality data, immune data was also collected. Most immune data correlated with mortality trends seeing the highest values around 48 hpc, while few treatment differences were observed. A culmination of these data allows us to conclude that coinfections between *F. covae* and virulent *A. hydrophila* increase mortality while causing the immunomodulation of proinflammatory genes at 48 hpc, and increased lysozyme activity at 12 hpc.

Future studies must investigate the effects of treatment options used during single infections on coinfections. Another promising avenue to explore could be looking deeper into coinfective infection dynamics due to portals of entry caused by other pathogens. This research documents the interaction between two bacterial pathogens exposed to channel catfish. Here mortality was greatly increased, even at half coinfection doses. This

work illustrates the potential for coinfections to cost production farmers millions, thus acting as a springboard for the further exploration of coinfection dynamics within the catfish industry.

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Tables

Table 3. Description of treatment groups, including which bacterium was administered, volume, and final dose for challenged channel catfish.

Treatment	Inoculum	Administered	Final Dose Administered
Single full dose *vAh*	virulent *A. hydrophila*	100 mL	2.3×10^7 CFU mL^{-1}
Single half does *vAh*	virulent *A. hydrophila*	50 mL	1.1×10^7 CFU mL^{-1}
Single full dose *F. covae*	*F. covae*	100 mL	5.2×10^7 CFU mL^{-1}
Single half does *F. covae*	*F. covae*	50 mL	2.6×10^7 CFU mL^{-1}
Full dose coinfection	virulent *A. hydrophila; F. covae*	100 mL; 100 mL	2.3×10^7 CFU mL^{-1}; 5.2×10^7 CFU mL^{-1}
Half dose coinfection	virulent *A. hydrophila; F. covae*	50 mL; 50 mL	1.1×10^7 CFU mL^{-1}; 2.6×10^7 CFU mL^{-1}
Controls	Phosphate-buffered saline	100 mL	NA

Figures

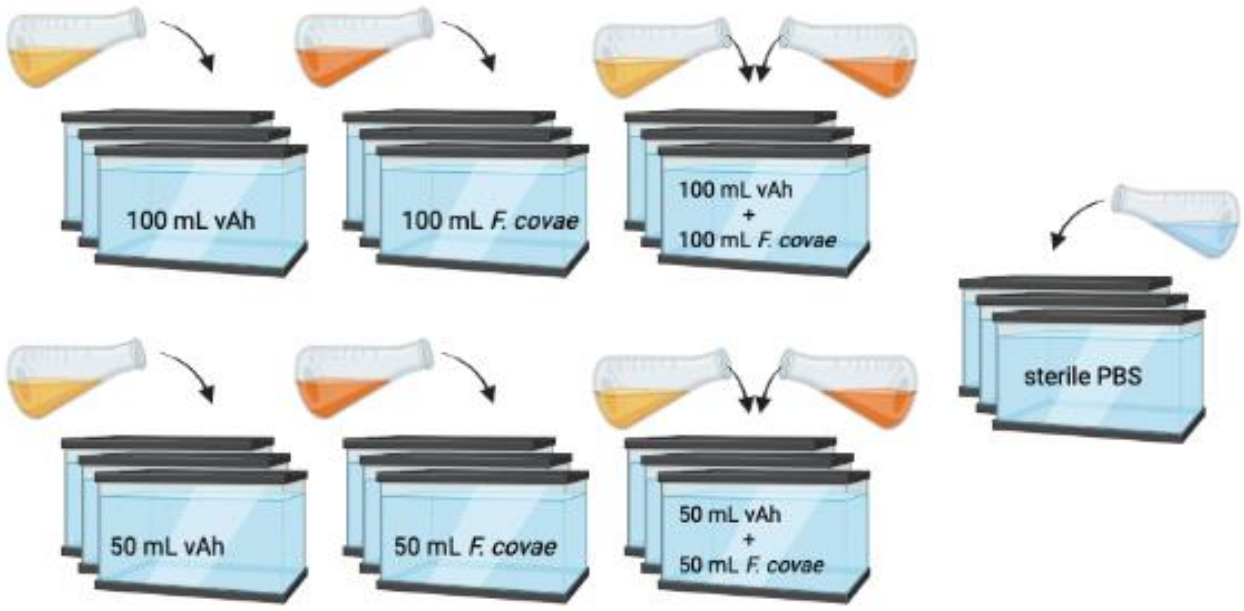

Figure 11. Graphic demonstrating experimental design to assess full and half doses of single and coinfective treatment groups.

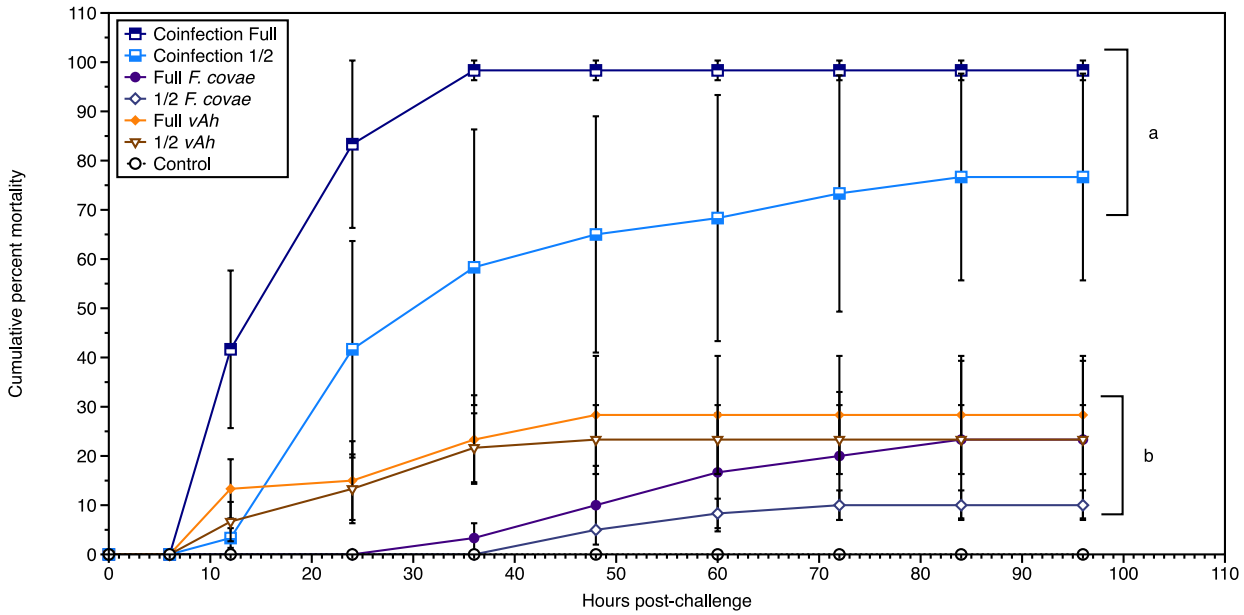

Figure 12. Cumulative percent mortality due to single infections of virulent *Aeromonas hydrophila* and *Flavobacterium covae* and coinfections from both pathogens throughout the trial (96 h). Each treatment group had three tanks (n=3). Bars represent the standard error of the mean for each day.

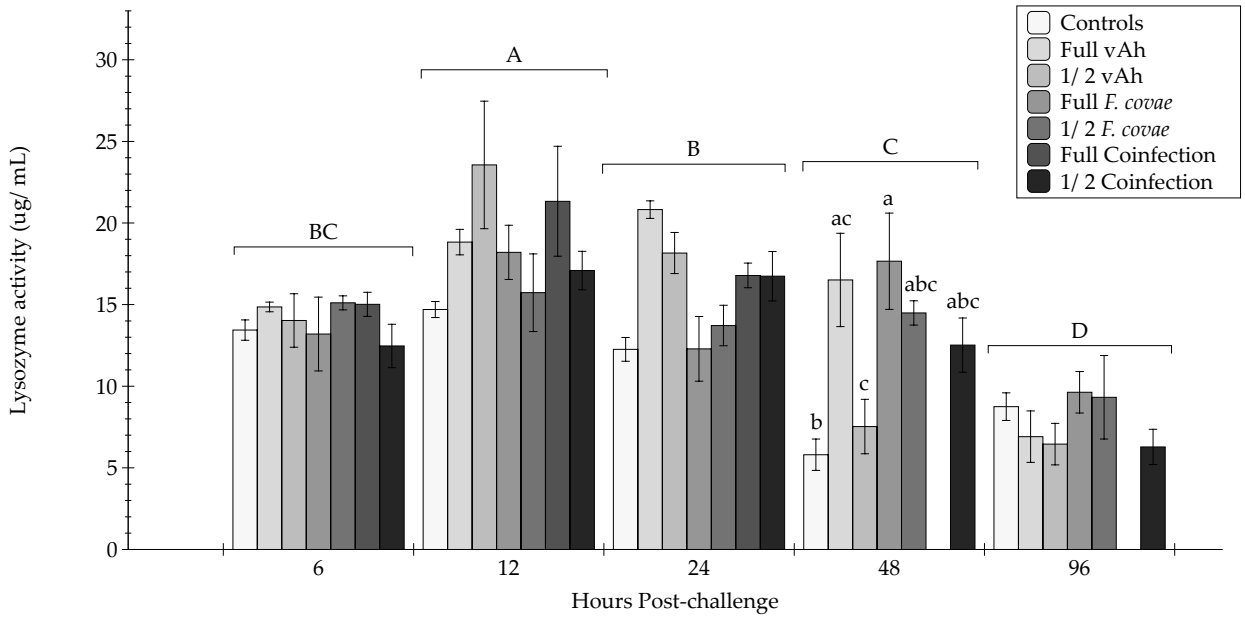

Figure 13. Lysozyme activity (mg mL^{-1}) in sera from sampled fish at 6, 12, 24, 48, and 96 hours post-challenge. Each treatment group was analyzed in triplicate (n=3). Capital letters indicate significant differences in activity between treatment time periods (6, 12, 24, 48, 96 hpc), and lowercase letters represent significance within treatment groups. Coinfected groups at 48 and 96 hpc are not included due to no surviving fish. Error bars represent the standard error of the mean for each treatment group.

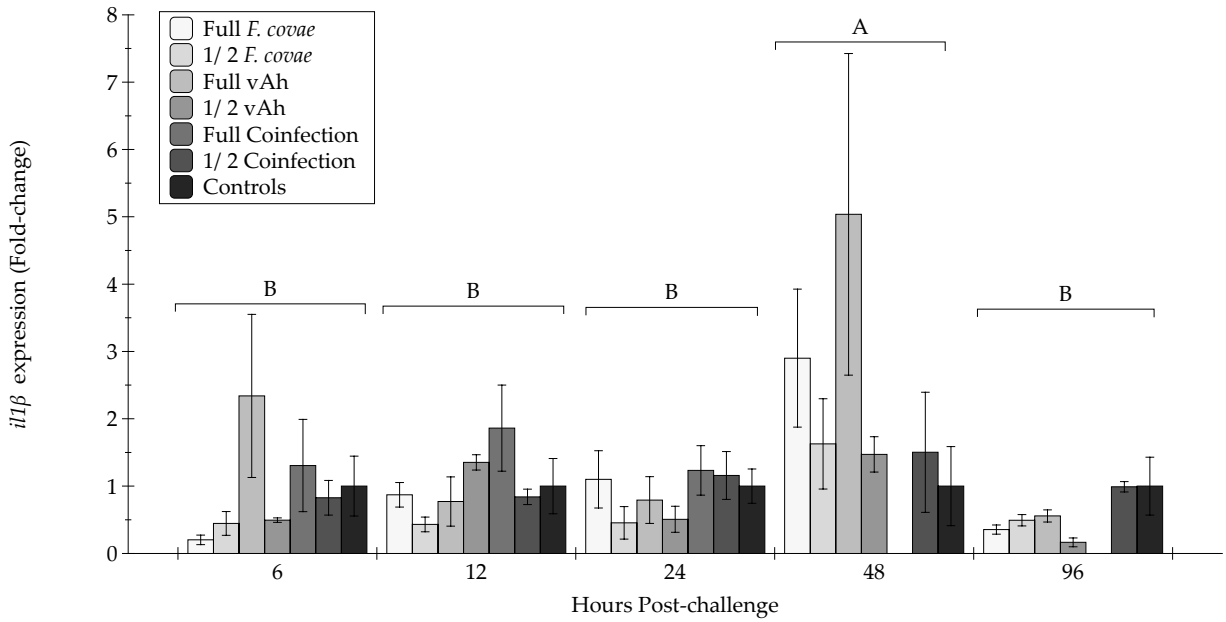

Figure 14. *il1b* expression (fold-change) was quantified from RNA extractions of anterior kidneys at 6, 12, 24, 48, and 96 hpc. Each treatment group was analyzed in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Bars represent the standard error of the mean for each treatment.

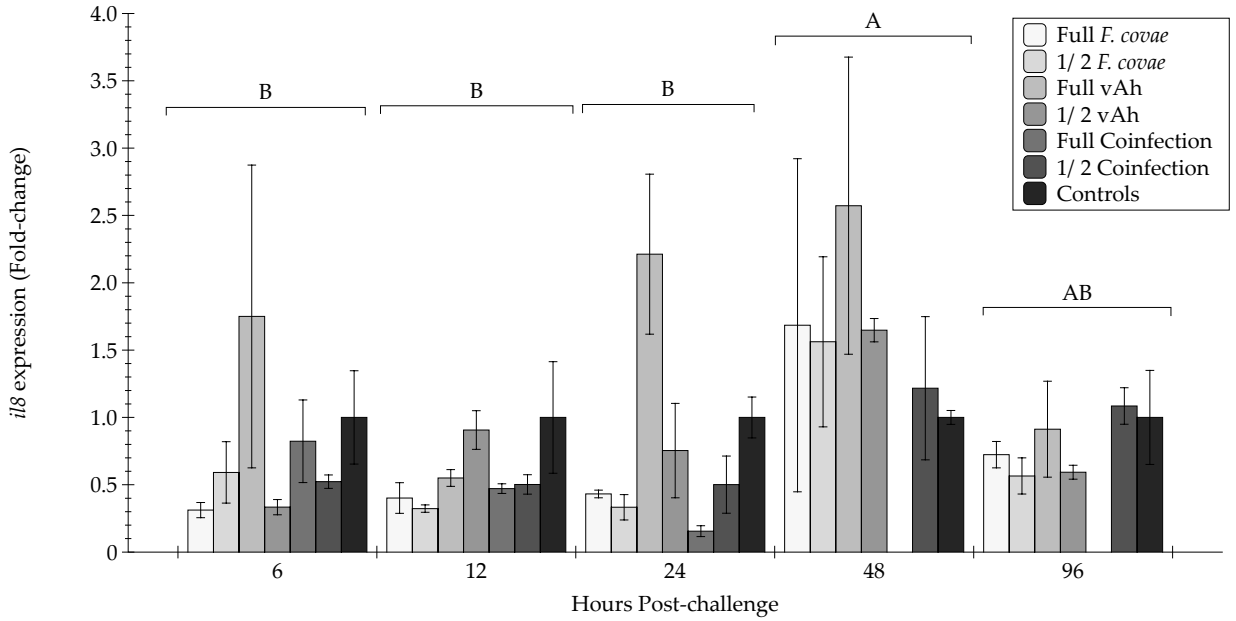

Figure 15. *il8* expression (fold-change) was quantified from RNA extractions of anterior kidneys at 6, 12, 24, 48, and 96 hpc. Each treatment group was analyzed in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Bars represent the standard error of the mean for each treatment.

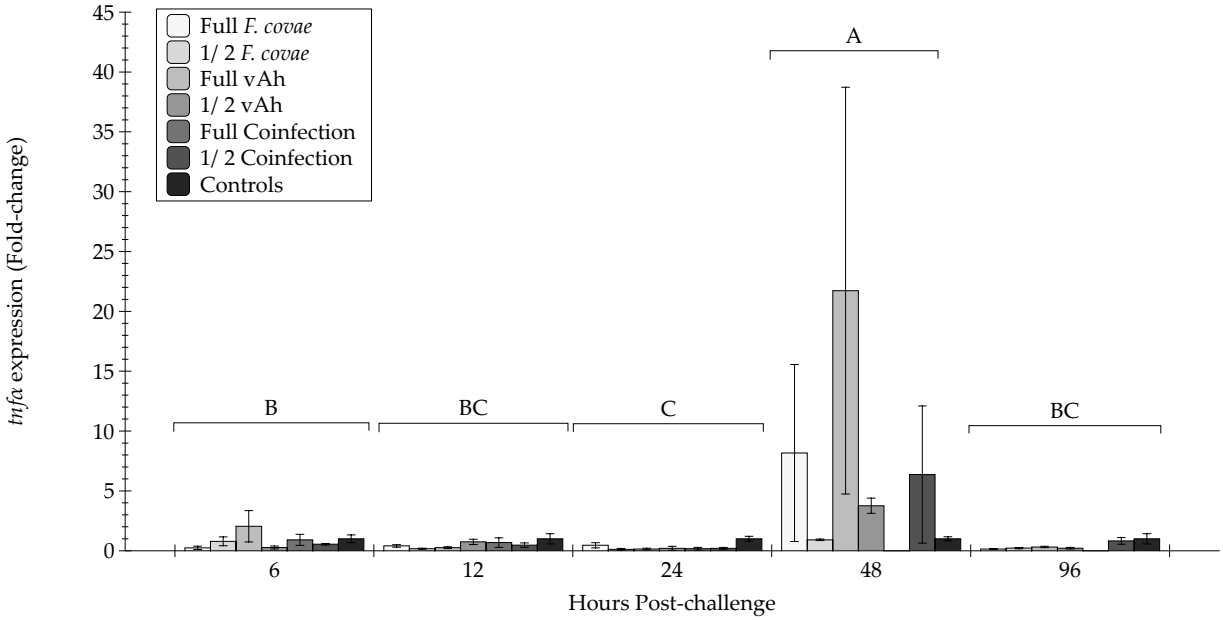

Figure 16. *tnfa* expression (fold-change) was quantified from RNA from anterior kidneys at 6, 12, 24, 48, and 96 hpc. Each treatment group was analyzed in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Bars represent the standard error of the mean for each treatment.

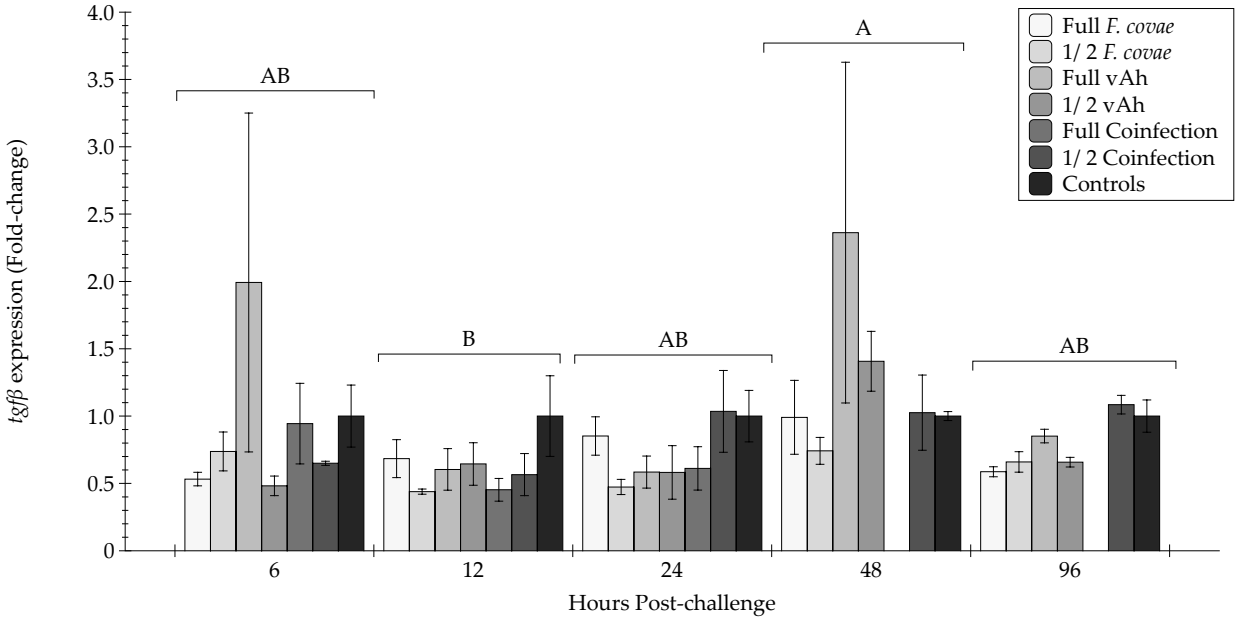

Figure 17. *tgfb* expression (fold-change) was quantified from RNA from anterior kidneys at 6, 12, 24, 48, and 96 hpc. Each treatment group was analyzed in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Bars represent the standard error of the mean for each treatment.

Figure 18. Images document catfish with clinical signs due to singly infected vAh: ocular hemorrhaging and hemorrhaging of all fins (A) and *F. covae*; skin discoloration, frayed fins, and damaged operculum (B).

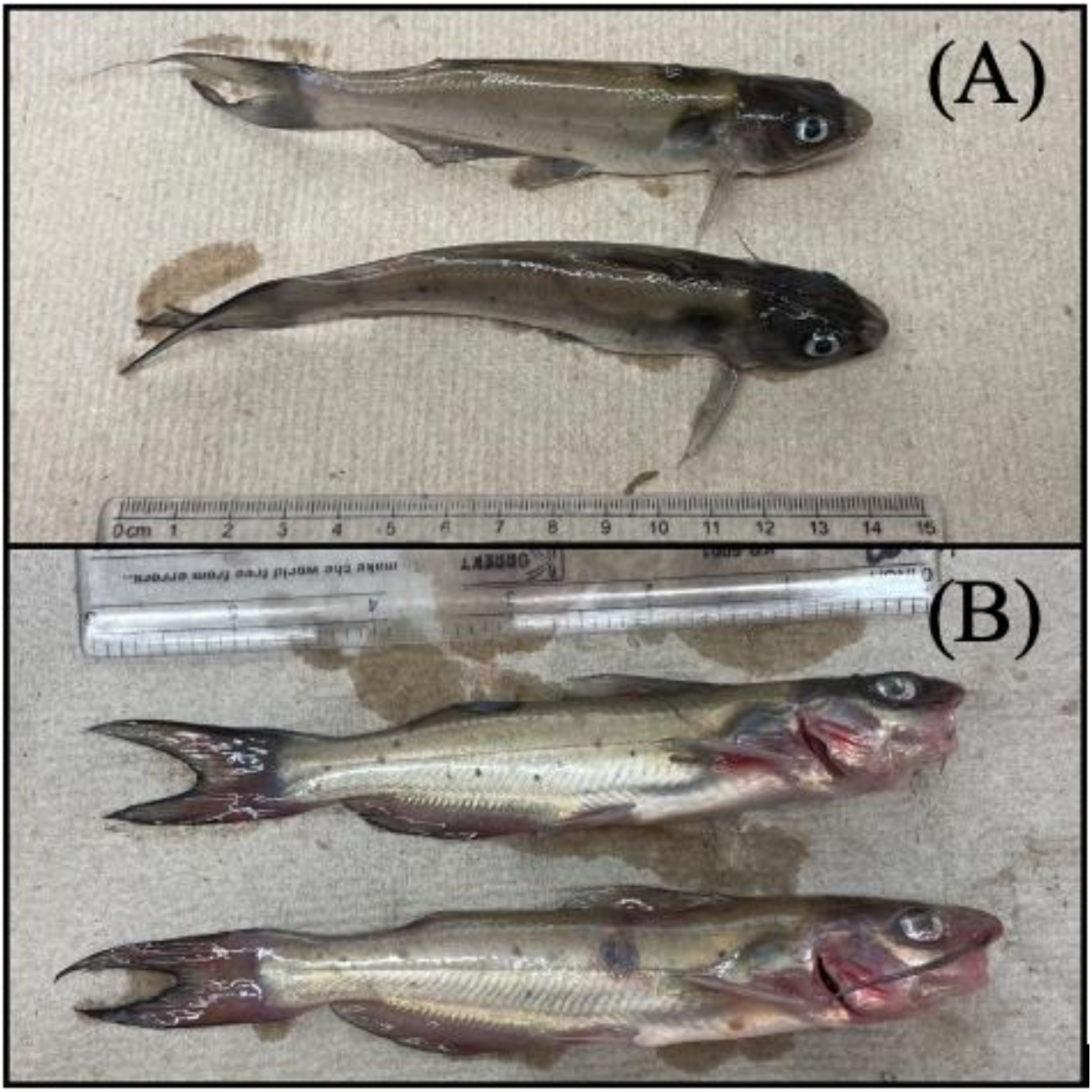

Figure 19. Images documenting catfish with clinical signs due to coinfection with *F. covae* and vAh during early-stage infection (12 hpc). Image (A) depicts discoloration of deceased fish (saddleback lesion), and (B) depicts mild and external hemorrhaging in fins and operculum.

Figure 20. Images documenting catfish with clinical signs due to coinfection with *F. covae* and vAh during later-stage infection (36 hpc).

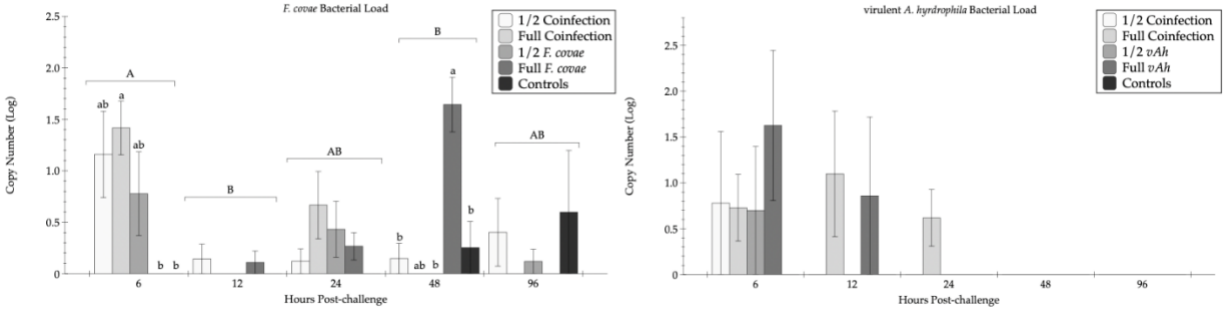

Figure 21. The bacterial load (log copy number) of treatment groups challenged with *F. covae* (ALG-00-530) and vAh (ML09-119). The log of the copy number corresponds to 5 ng of input DNA for each sample. Each treatment group was analyzed in triplicate (n=3). Capital letters indicate significant differences in quantity between treatment time periods, and lowercase letters represent significance within treatment groups. Bars represent the standard error of the mean for each treatment.

Chapter 5:

Future Coinfection Research in Catfish

5.1 Future Research

Future research is crucial in solving and avoiding the devastating impacts coinfections can create. These trials may be repeated with higher replication or pathogen variants to provide more insight. Higher replication will confirm observed trends by demonstrating more significant differences. Each treatment group only had three replicates, while typically immersion trials have at least five replicates. Observing significant differences with just the three replicates indicates a high effect coinfections have on mortality, and higher replicates will not only confirm this, but provide the ground work needed to bring light and urgency to the coinfection crisis. The trial design could also be modified to investigate how portals of entry impact the severity of infection. It is known some pathogens (*F. covae*, *Bolbophorus spp*., *Henneguya ictaluri*) can cause lesions whether that be externally or though the gills. These exposures can allow other opportunistic pathogens in enter the host directly potentially causing more severe mortality and clinical effects. By modifying trial designs to investigate other coinfections, we broaden the scope and begin to truly unravel coinfection dynamics between not only bacterial pathogens but parasitic ones as well.

A critical step to solidify and prove the deadly impact coinfections cause is evaluating coinfections in production settings to see if the same effects documented during these experimental trials still exist. Pond trials play a critical role in applying data observed in these laboratory trials to production ponds. Farmers must see the true effects of coinfections and be presented with possible solutions. Once this dynamic has been thoroughly assessed, testing antimicrobial treatments under experimental settings must be

conducted to test if single infection treatment methods are just as efficacious during coinfections. This would deliver the last piece of the coinfection puzzle by providing evidence of its impact and a solution for how best to mitigate it.

5.2 Conclusion

Bacterial coinfections in channel catfish between vAh, *E. ictaluri*, and *F. covae* have, until now, never been assessed. Coinfections are a plague within multiple aquaculture industries, but so little is known about their infection mechanics and how the infection is to be mitigated. Bacterial diseases cause tremendous economic losses. Within the catfish industry, losses are typically associated with single pathogens and do not take into account losses due to coinfections. This leaves researchers scrambling to understand the true prevalence and interactions between pathogens during coinfections. The need for this information is crucial; with it, treatments may be more effective and efficient. At this moment, no current antibiotic treatments deemed successful to treat single pathogens have been evaluated during coinfections. However, before treatments can be assessed, infection dynamics must be evaluated. This thesis aimed to begin assessing these bacterial coinfections in channel catfish to provide evidence of the potentially deadly impact they pose in production and to stress the need for treatment evaluations.

To do this, closer looks at mortality rates, innate and adaptive immune attributes, and bacterial interactions will provide a clear explanation as to what exactly is causing these investigated parameters to be augmented. Evaluating mortality between coinfected treatments and single-exposed treatments would demonstrate the severity of coinfections.

Further use of transcriptomics to map immune attributes may also be able to answer whether each coinfection causes synergistic or antagonistic effects. Understanding if the pathogens inhibit one another or work together to obtain host resources allows researchers to recognize potential pathways that could be modified to mitigate each pathogens effects. All trials documented herein demonstrated that coinfections augment mortality and the innate immune response was upregulated during days where the most mortality was occurring; however, this is the tip of the iceberg when fully understanding coinfections in production systems and should act as the foundation of future studies.